THE CONTROL OF EMBRYOGENIC COMPETENCE IN ALFALFA (*Medicago falcata* L.)

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AUTHOR'S DECLARATION

This thesis is entirely my own work and has at no time been submitted for another degree.

.....
(YAN ZHOU)

I certify that this statement is correct.

.....
(ADRIAN SLATER)

DEDICATION

This thesis is dedicated to: my husband Yi-Peng,

my parents and my son Feng.

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ABBREVIATIONS

ABA	abscisic acid
ABP	auxin-binding protein
AGP	arabinogalactan proteins
AMP	adenosine monophosphate
ATP	adenosine triphosphate
Asp	asparagine
BAP	N-6-benzylaminopurine
Bar	gene encoding phosphinothricin acetyltransferase
B_5	Gamborg's B_5 medium
BCIP	5-Bromo-4-Chloro-3-Indolylphosphate
B_5h	Brown and Atanassov (1985)
ble	gene encoding bleomycin resistance
Boi2Y	Blaydes medium with 100 mg L^{-1} inositol and 2 g L^{-1} yeast extract
bp	base pairs
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
BZIP	basic zipper
CAK	CDC-activating kinase
CaMV	Cauliflower Mosaic Virus
cat	gene encoding chloramphenicol acetyltransferase
CDK	cyclin-dependent kinase

cDNA	complementary deoxyribonucleic acid
DAPI	4',6-diamidino-2-phenylindole
dATP	deoxyadenosine triphoshate
dCTP	deoxycytidine triphosphate
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanodine triphosphate
2,4-D	2,4-dichlorophenoxyacetic acid
DIG	digoxigenine triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
dUTP	deoxyuridine triphosphate
dTTP	deoxythymidine triphospate
EC	embryogenic cell
E. coli	*Escherichia coli*
EDC	embryogenic determined cell
EDTA	ethylenediamine tetraacetic acid
EP	extracellular protein
ER	endoplasmic reticulum
FAA	50% ethanol, 5% acetic acid and 10% formalin (3.7% formaldehyde)
G	guanine
GA	gibberellic acid
GFP	green fluorescent protein

G0	quiescent phase
G1	before DNA synthesis
G2	after DNA synthesis
Glu	Glutamine
GusA(*uidA*)	gene encoding β-glucuronidase enzyme
GUS	β-glucuronidase
h	hour
HD-Zip	homeodomain leucine-zipper
hpt	gene encoding hygromycin phosphotransferase
HSP	heat shock protein
IAA	indole-3-acetic acid
IEDC	indirect embryogenesis determined cell
ipt	isopentenyl transferase gene
IPTG	isopropyl-β-D-thiogalatoside
kb	kilo base
kD	kilo Dalton
Km	kanamycin
LB	Luria-Bertani
LEA	late embryogenesis abundant
Lys	lysine
M	mitosis
MAP	mitogen activated protein
MAPK	mitogen activated protein kinase

MCS	microcallus suspensions
mRNA	messenger RNA
MS	Murashige and Skoog medium
MSH	Murashige and Skoog with 22.6 μM 2,4-D and 4.7 μM kinetin medium
MW	molecular weight
NAA	naphthalene acetic acid
nos	gene encoding nopaline synthase
npt II	neomycin phosphotransferase II gene
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEDC	pre-embryogenic determined cell
PEG	poly-ethylene glycol
PEM	pro-embryogenic mass
PGR	plant growth regulator
RACE	rapid amplification of cDNA end
RAM	root apical meristem
RAP-PCR	RNA arbitrarily primed PCR
Ri	root-inducing plasmid
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
rpm	revolutions per minute
S	synthetic phase

SAM	shoot apical meristem
SDS	sodium dodecyl sulphate
SERK	somatic embryogenesis receptor-like kinase
SSC	standard saline citrate
T	thymine
TAE	Tris-acetate-EDTA buffer
Taq	*Thermus aquaticus*
T-DNA	transfer DNA
TDZ	thidiazuron
TE	Tris-EDTA buffer
Thr	threonine
Ti	tumour-inducing plasmid
TIBA	2,3,5 tri-indobenzoic acid
TL-DNA	left T-DNA
TR-DNA	right T-DNA
Tris	Tris(hydroxymethyl)-aminomethane
Tyr	tyrosine
U	unit
UTP	uridine triphosphate
vir	virulence genes
v/v	volume/volume ratio
w/v	weight/volume ratio
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D glucuronide

ABSTRACT

Somatic embryogenesis is a process by which somatic cells undergo a developmental sequence similar to that observed in zygotic embryos. This process is not only an important plant propagation technique, but it also provides an essential tool for basic research into plant embryo development and other aspects of plant physiology. One of the basic features of the initiation of somatic embryogenesis is the reactivation of the cell cycle and subsequent cell division in differentiated plant cells. The initiation of somatic embryogenesis effectively involves resetting an entire ontogenic program and presumably requires a substantial reprogramming of gene expression patterns. The reactivation of cell division may be an essential prerequisite for this genetic reprogramming.

In this project, transgenic alfalfa plants carrying *Arabidopsis cdc2a* and, *CycB1;*1 promoter::*gusA* constructs, were used to investigate the activation of cell division during the induction of somatic embryogenesis. Promoter fragments of a sugar beet cdc2-like protein kinase gene (*Bvcrk1-17, 26*) fused to the *gusA* reporter gene were also introduced into alfalfa plants by *Agrobacterium*-mediated transformation, to investigate their potential as molecular markers for re-entry into the cell division cycle. Histochemical assays of β-glucuronidase (GUS) activity were carried out to investigate cell division activity patterns during direct somatic embryogenesis. The cell cycle inhibitors oryzalin and hydroxyurea were used to determine the relationship between the induction of cell division and somatic embryogenesis. The role of 2,4-D in the induction of cell division and somatic embryogenesis was also examined using these transgenic alfalfa lines. The expression patterns of the cell cycle promoter constructs in response to 2,4-D were compared with their behaviour in transgenic tobacco under the same conditions. Preliminary studies indicated that the response to 2,4-D induction treatment in activation of cell division was different in these two systems.

The expression patterns of a group of clones isolated from induced alfalfa leaf explants by subtractive cloning were determined by dot blot array hybridisation. Some of these genes were found to be expressed during the very early stages of

induction of somatic embryogenesis. Certain genes associated with signal transduction (protein phosphatase [PP2C]; lysophospholipase, [PLP]) and gene regulation (bZIP and HD-Zip class transcription factors) were selected for further characterisation. 5' RACE and 3' RACE products of the original clones were subcloned in order to extend the DNA sequence information. These DNA sequence data were used to search the sequence databases by BLAST homology searching. These results indicated similarity to genes which function as regulatory elements during plant development.

The HD-Zip transcription factor gene (*Mfhb-1*) was of particular interest because similar genes have been associated with somatic embryogenesis in other species. One feature of this gene was the presence of a highly conserved uORF sequence. In order to characterise the role of this gene in somatic embryogenesis, constructs carrying different fragments of the gene in sense and antisense orientations were established and introduced into alfalfa plants via *Agrobacterium*-mediated transformation and transformants with each of the constructs were successfully regenerated. The phenotypic effects of overexpression and antisense expression of the HD-Zip transcription factor gene during regeneration of the transformants and subsequent direct somatic embryogenesis in suspension culture, provided some insight into its biological function. It is proposed that the HD-Zip transcription factor gene *Mfhb-1* may be involved in the control of the auxin response (particularly auxin polar transport) during somatic embryogenesis in alfalfa. Expression of the HD-Zip gene enhances somatic embryogenesis, and the uORF may serve to enhance the expression or activity of the HD-Zip protein.

CONTENTS

CHAPTER 1 GENERAL INTRODUCTION

1.1 The importance of somatic embryogenesis

The capacity of somatic plant cells in culture to form embryos by a process resembling zygotic embryogenesis is one of the most remarkable features of plants. This phenomenon exemplifies the concept of totipotency, which is proposed to be a property of most multicellular organisms. Totipotency in higher plant means that single cells other than zygotes are able to divide and differentiate to produce adult plants. Under appropriate condition, some cells are able to develop along various pathways to form embryos without fertilisation, which are collectively referred to as apomixis (for reviews, Koltunow, 1993; Koltunow *et al.*, 1995). Somatic embryogenesis represents one type of non-zygotic embryo developmental pathway. In this process somatic cells develop through the stages of embryogeny to form whole plants without gamete fusion (Merkle *et al.*, 1990). For dicotyledonous plants the typical progression of embryo formation follows four stages: globular, heart, torpedo and cotyledon stage. Other types of non-zygotic embryo development include microspore embryogenesis, in which haploid embryos are formed from male gametic cells, and gametophytic apomixis, in which an asexual reproductive process can occur from the somatic cells in the ovule so that embryos are formed without fertilisation (Koltunow, 1993; Koltunow *et al.*, 1995; Calzada *et al.*, 1996).

Somatic embryogenesis can occur naturally in several genera such as *Bryophyllum* (Yarbrough, 1932) and *Malaxis* (Taylor, 1967), where somatic embryos form spontaneously on the leaf margins; and it can also be induced by experimental manipulation. By 1995, tissue culture conditions for the induction of somatic embryogenesis had been described for over 200 plant species (Raemakers *et al.*, 1995), and increasing numbers of protocols were published in the last few years. Since the first observation of somatic embryo formation in carrot cell suspension cultures was described by Steward *et al.* (1958) and Reinert (1959), this phenomenon has been reported in over 300 plant species (reviewed by Bajaj, 1995). Somatic embryogenesis offers a wide range of applications for agricultural crop improvement, as it is capable of providing a larger number of plants in a shorter period of time than other approaches. It also offers

opportunities for regeneration of genetically modified plants by genetic transformation, and for somatic hybridisation and *in vitro* mutant induction and selection. Moreover, somatic embryogenesis is an important tool for basic research in understanding the molecular and cellular bases of developmental plasticity in plants (Gaj *et al.*, 2004 in press; Fehér *et al.*, 2003). In higher plants, the events of fertilisation and subsequent embryo development normally occur deep within the maternal tissue. The early stage embryo is minute and is surrounded by both endosperm and maternal cells. Although the morphological description of embryo development has been extensively recorded through microscopy, molecular and biochemical analysis of early embryogenesis is hampered by this physical inaccessibility. The development of somatic embryogenesis closely resembles that of zygotic embryos both morphologically and temporally, so somatic embryogenesis provides an appropriate model system *in vitro* to study the regulation of gene expression during the earliest developmental events in higher plants, from the development of the fertilised zygote into a mature embryo. This model system is not limited by tissue quantity or accessibility.

Interpreting the molecular processes that occur when somatic cells become embryogenic is considered to be one of the biggest steps towards understanding the mechanisms that control of cell differentiation and development in higher eukaryotes. Recent advances in molecular biological techniques and the exponential increase in information following the recent sequencing of the *Arabidopsis* genome have created completely new opportunities for a more comprehensive study of this developmental transition. The success of these studies will contribute to a better understanding of the underlying cellular and molecular mechanisms.

1.2 The differences between somatic embryogenesis and zygotic embryogenesis

In Angiosperms, double fertilisation generates the embryo and the endosperm simultaneously, and it is their joint development that results in a viable seed. The characteristic developmental stages of dicotyledon embryogenesis include the globular, heart, torpedo and cotyledon stages. In monocotyledonous plants, for example maize (*Zea mays* L.), these stages are globular and club-shaped or ovoid stages followed by

development of a bipolar embryo axis attached to the scutellum (the embryo's storage organ), the cotyledon-stage (Emons and Kieft, 1995).

In general, somatic embryogenesis and zygotic embryogenesis share striking similarities, although some slight differences exist between them. Anatomical comparisons of zygotic and somatic embryos (Halperin and Jensen, 1967; Street and Withers, 1974) showed that, except for subtle differences in the patterns of division in early pre-globular forms and in the morphology of the suspensor region, the morphogenetic processes are nearly identical (Racusen and Schiavone, 1990). The fact that structurally and developmentally normal embryos can develop from somatic cells demonstrates that plant somatic cells contain the entire set of genetic instructions necessary to create a complete and functional plant. It also shows that the genetic programme for embryogenesis and its elaboration can function totally in the absence of gene products from the maternal environment. The zygote is intrinsically embryogenic but this is not the case with somatic cells. The latter requires the induction of embryogenic competence in cells which are not naturally embryogenic (Dodeman *et al.*, 1997). In this process, the components in the medium (e.g. plant growth regulators) may somehow mimic natural signals to initiate embryogenesis (Zimmerman, 1993). In practice, the successful induction of somatic embryos and subsequent recovery of viable plants is not routine or efficient for the majority of species. Molecular and genetic studies revealed that embryo development is comprised of a variety of different stages involving specific patterns of gene expression (Goldberg *et al.*, 1989; Hughes and Galau, 1989; Kermode, 1990). The more closely the pattern of somatic embryo gene expression matches that of zygotic embryos, the greater the chance of obtaining highly efficient regeneration systems. Such normalisation of gene expression patterns will be achieved through the optimisation of media and culture protocols for each individual stage of embryo development (Merkle *et al.*, 1995).

1.3 Transition of somatic plant cells to an embryogenic state

Somatic embryogenesis provides a unique experimental model for studying the mechanisms of de-differentiation and re-differentiation of plant cells. In the last few years the application of modern experimental techniques, as well as the characterisation of *Arabidopsis*

embryogenesis mutants, have resulted in the accumulation of novel data about the acquisition of embryogenic capabilities by somatic plant cells. During this somatic-to-embryogenic transition, cells have to dedifferentiate, activate their cell division cycle and reorganise their physiology, metabolism and gene expression patterns (Fehér, *et al.*, 2003).

In order to gain a better understanding of the early events leading to somatic embryo formation, the concepts of competence, induction and determination originally proposed by animal biologists have been used by plant scientists to interpret their observations (Ammirato, 1985; 1987; Christianson, 1987; Wareing, 1978; Meins, 1986; McDaniel, 1984; Lyndon, 1990; Yeung, 1995).

Competence is the capacity to respond to specific signals, e.g. environmental, chemical, or other manipulated treatments, and to do so in a consistent manner (Meins, 1986; McDaniel, 1984 and Lyndon, 1990). The term embryogenic competence is helpful for describing the relative state of induction of somatic embryogenesis from tissue cultures (Carman, 1990). Somatic embryos are formed by embryogenic cells, which arise from the somatic cells of an explant, callus or suspension cells. Embryogenic cells have the ability to form somatic embryos without stimulation by external factors (De Jong *et al.*, 1993). Whether these competent cells will indeed express their embryogenic character depends on the tissue culture environment (Emons, 1994), such as hormone balance, osmotic condition, sucrose status, amino acid and salt concentrations (Armstrong and Green, 1985; Rhodes *et al.*, 1986). Competence varies by degree and may depend on the activity of genes "responsible" for embryogenesis. In theory all healthy, undamaged somatic cells are totipotent; in practice only a few of them are sensitive to embryogenic induction factors and capable of undergoing somatic embryogenesis. Competence is generally highest in the non-meristematic tissues of immature embryos, mature embryos, seedlings, floral structures and very young tissues (Carman, 1990; Zimmerman, 1993).

Induction occurs when a signal produces a unique developmental response from competent tissue (McDaniel, 1984). The differentiated fate of plant cells, dependent on positional information and developmental signals, can be easily altered under *in vitro* conditions. Drastic changes in the cellular environment, such as exposing wounded cells or tissues to sub-optimal nutrient or hormone supply generate significant stress effects. The response to stress conditions is determined by two parameters: the level of stress and the physiological

state of the cells. If the stress level exceeds cellular tolerance, the cells die. In contrast, low levels of stress enhance metabolism and induce adaptation mechanisms (Lichtenthaler, 1998). Adaptations include the reprogramming of gene expression, as well as changes in the physiology and metabolism of the cells (Fehér *et al.*, 2003). These procedures were accompanied by increased expression of diverse stress-related genes, evoking the hypothesis that somatic embryogenesis is an adaptation process of in vitro cultured plant cells (Dudits *et al.*, 1995).

Determination is a process by which the developmental fate of a cell or group of cells becomes fixed and is limited to a particular developmental pathway (Ammirato, 1985). The developmental potential of cells/tissues/organs becomes more restricted as determination proceeds, and involves stable changes in the phenotype (Meins, 1986). Although these events are usually tightly coupled and may be impossible to separate, the usefulness of these terms is that they allow for a systematic way of thinking about early events in somatic embryogenesis (Dudits *et al.*, 1995).

The transition from somatic cells to embryogenic cell stages involve characteristic, although overlapping, phases of dedifferentiation, cell division, acquisition of competence, induction and determination of the embryogenic cell fate (Fehér, *et al.*, 2003). After this transition, the cell or cell culture is called "embryogenic". Cells in this transition between the somatic and embryogenic cell state are defined as being in a "competent cell state" (De Jong *et al.*, 1995; Komamine *et al.*, 1990; Toonen *et al.*, 1994).

1.4 Systems for studies of cellular and molecular basis of somatic embryogenesis

1.4.1 Direct and indirect systems

Under *in vitro* conditions, two basic patterns of induction have been generally recognised: direct and indirect embryogenesis. In direct somatic embryogenesis, embryos can form directly from an organised tissue such as leaf or stem segment, from protoplasts or from microspores. Indirect somatic embryogenesis requires re-determination of differentiated cells and the acquisition of the embryogenic state via an intermediate callus or suspension culture step prior to the initiation of embryo development (Ammirato, 1987; Williams and

Maheswaran, 1986). However, these terms, as will be discussed below, may now be redundant. Central to our understanding of embryogenesis has been an effort to describe the routes taken by the reprogramming process.

'Direct' embryogenesis and 'indirect' embryogenesis were first distinguished by Sharp *et al.* (1980). The term 'direct' was applied to explants which undergo a minimum of proliferation before forming somatic embryos. The term 'indirect' was applied to explants which undergo an extensive period of disorganised proliferation before somatic embryos can develop. Direct embryogenesis tended to occur in explants consisting of young plants such as embryos or seedlings or in tissues newly derived from meristems. Indirect embryogenesis was thought to be characteristic of mature organs in which cells must go through several cell cycles in order to achieve the embryogenic or 'determined' condition (Williams and Maheswaran, 1986; Halperin, 1995).

Merkle *et al.* (1990) have provided an excellent discussion on the distinction between 'direct' and 'indirect' somatic embryogenesis: In their view, the linguistic distinction between "direct" and "indirect" is misleading. In practice these terms define opposite ends of a continuum whose intermediate regions may be difficult to quantify (Dudits *et al.*, 1995 p224). To distinguish patterns of embryogenesis as direct or indirect based simply on intercalation of mitotic cycles between explant and embryo organisation is, in physiological terms, an oversimplication. The most meaningful way to define "direct" and "indirect" appears to be with reference to the epigenetic state of explant cells. Thus, somatic cells which are themselves embryonic, or not far removed from embryonic, are generally more easily induced to undergo somatic embryogenesis than differentiated vegetative cells. Highly differentiated cells appear to require major epigenetic changes, making the initiation of embryogenesis less direct. In these terms, the directness of embryogenesis is measured as epigenetic "distance" of explant cells from the embryonic state.

Sharp *et al.* (1980, 1982) and Evans *et al.* (1981) classified cells undergoing somatic embryogenesis as either 'induced embryogenic determined cells' (IEDCs) or 'pre-embryogenic determined cells' (PEDCs). IEDCs were used to describe embryonic cells that had originated from nonembryonic cells. IEDCs are the products of an epigenetic switch to the embryogenic state in culture. While PEDCs were used to describe cells from

plant embryos that already express an embryonic gene expression programme (Merkle *et al.*, 1995). PEDCs are epigenetically embryonic at explanting, e.g., cells of a zygotic embryo. Once obtained, both IEDCs and PEDCs are functionally equivalent (Merkle *et al.*, 1991,1995) and both may be referred to as 'EDCs' for 'embryogenic determined cells' (Williams and Maheswaran, 1986) or simply, 'ECs' for 'embryogenic cells' (Carman, 1990; Merkle *et al.*, 1990). The latter term seems to be more acceptable, because the formation of a somatic embryo is not an inevitable fate for an EC, indicating an existing plasticity in the cells not adequately conveyed by the term 'determined' (Carman, 1990). Treatment to obtain somatic embryogenesis is thus dependent on whether the explant tissue contains PEDCs or non-ECs. In the first case, a stimulus for cell division may be sufficient for the formation of a somatic embryo on a tissue explant, in a process referred to as direct embryogenesis, as the somatic embryos appear to arise directly from the explant tissue. In contrast, non-EC tissue must undergo several mitotic divisions in the presence of an auxin during the induction of the EC state. Cells resulting from these mitotic division are manifested as a callus, and the term indirect regeneration is used to indicate that a callus phase intervenes between the original explant and the appearance of somatic embryos (Merkle *et al.*, 1995). In practice, an explant tissue may fall anywhere in a continuum between a PEDC state and a non-EC state, depending on its age. This influences the degree of the ease and the 'directness' with which somatic embryos may be induced (Parrott *et al.*, 1991). This may explain why gradients in embryogenic capacity have been observed in various explant tissues (e.g., Tisserat *et al.*, 1979; Hartweck *et al.*, 1988; Pretova and Williams, 1986; Young *et al.*, 1987; Ghazi *et al.*, 1986; Ranch *et al.*, 1985; Barwale *et al.*, 1986; Karunaratne *et al.*, 1991; Vasil, 1987; Williams *et al.*, 1990; Dolezelova *et al.*, 1992).

More recently the definitions of direct and indirect embryogenesis were clarified by applying the term 'unorganised' and 'embryogenic" cell or cells (De Jong *et al.*, 1993). 'Unorganised' was employed to describe suspension cultures whereas 'embryogenic cell or cells' was used to describe only those cells that have completed the transition from a somatic cell or cells to a state where no further externally applied stimuli are necessary to produce the somatic embryo. Following from this, a culture or tissue with a variable number of cells in it that have responded to external stimuli will be called an

'embryogenic culture or tissue'. Depending on the experimental conditions, the ratio of embryogenic to total cells under these conditions can vary between zero and the theoretical maximum of 1. According to this, the difference between direct and indirect somatic embryogenesis is no longer of importance. Direct embryogenesis on explants or indirect embryogenesis on callus or clusters of embryogenic cells in suspension cultures may therefore represent different sides of the same coin (Williams and Maheswaran, 1986).

1.4.2 Model systems

Several embryogenic systems have been employed for studies of the molecular basis of somatic embryogenesis. They are: carrot (*Daucus carota* L.) cell suspension cultures (Borkird *et al.*, 1988; Komamine *et al.*, 1990; Racusen and Schiavone, 1990), carrot (*Daucus carota* L.) hypocotyls direct somatic embryogenesis system (Tokuji *et al.*, 1993, 1996; Masuda *et al.*, 1995), *Arabidopsis thaliana* direct and indirect somatic embryogenesis system (O'Neill and Mathias, 1993; Huang and Yeoman, 1984; Wu *et al.*, 1992; Luo and Koop, 1997), alfalfa (*Medicago sativa* L.) microcallus suspension protoplast cultures (Dudidt *et al.*, 1991), alfalfa (*Medicago sativa* L.) mesophyll protoplast cultures (Dudidt *et al.*, 1991; Kao and Michayluk, 1980; Dijak *et al.*, 1986) and alfalfa (*Medicago falcata* L.) explant direct somatic embryogenesis system (Denchev *et al.*, 1991 a, b, 1993).

1.4.2.1 Carrot cell suspension cultures

Carrot (*Daucus carota* L.) cell suspension cultures are the most extensively studied embryogenic systems and they are also considered to be the ideal model for studies of the molecular basis of somatic embryogenesis (Borkird *et al.*, 1988; Komamine *et al.*, 1990; Racusen and Schiavone, 1990). In carrot cultures, somatic embryos are induced from cultured callus cells. The main steps of the process generally involves: (1) the establishment of a callus cell line from small hypocotyls pieces cut from sterilely germinated individual seeds, (2) the selection of an embryogenic subpopulation of the

cultured cells through sieving or gradient fractionation, (3) the removal of auxin from the culture medium, and (4) the dilution of the cells to a relatively low density (Zimmerman, 1993) (Figure 1.1). It is generally accepted that in carrot cultures the embryogenic state is already induced when the cell line is established and the proembryogenic masses (PEMs) are propagated in the presence of 2,4-D (Dudits *et al.*, 1991). In this process, cells continually proliferate; they successively divide and grow and can form secondary embryos, but do not differentiate into the organs of the plant body. Calli may be held in this condition for years by repeated subculturing, though their embryogenic potential generally decreases with time. These proliferating calli or suspensions have often been described as 'undifferentiated', which is as De Jong *et al.* (1993) correctly points out, is a confusing use of terminology, because in embryogenic callus and suspension cultures subpopulations of morphologically and biochemically different cell types occur (Emons *et al.* 1992; Pennell *et al.*, 1992). De Jong *et al.* (1993) introduced the term 'unorganised'. However, in embryogenic calli and PEMs in suspension cultures there are always several types of 'organised' cells (Emons, 1994). In fact the very young pre-globular somatic embryos are already present in these cultures (Emons *et al.*, 1992; Emons, 1994). In addition to the crucial role of the exogenous auxins in generating embryogenic potential, 2,4-D is also thought to inhibit the progression of embryo development in carrot multicellular colonies. Therefore the completion of embryogenesis requires hormone-free culture conditions or low cell density (Sung and Okimoto, 1981, Dudits *et al.*, 1991; Zimmerman, 1993). Recently, Osuga *et al.* (1997) reported that partial replacement of medium in carrot suspension cultures increased the frequency of somatic embryogenesis at high embryo density.

The proembryogenic nature of carrot suspension cultures makes it difficult to determine the time of commitment of somatic cells towards embryogenesis (Dudits *et al.*, 1991. 1995). So alternative experimental systems are required for a comprehensive study of the activation events at the initiation of embryogenic development in somatic cells.

1.4.2.2 The carrot hypocotyls direct somatic embryogenesis system

A new system for carrot (*Daucus carota*) direct somatic embryogenesis was developed. In this system, primary explants of carrot hypocotyls were exposed to Murashige-Skoog (MS) culture medium containing 2,4-D, for a period of time (1-24 hours, depending on the concentration of 2,4-D) and then transferred to MS medium lacking 2,4-D. (Ohashi *et al.*, 1993; Tokuji *et al.*, 1993, 1996; Masuda *et al.*, 1995). Using this approach, somatic embryos were formed directly via the formation of cluster cells from epidermal cells without intervening callus and cell suspension culture phases (Masuda *et al.*, 1995).

1.4.2.3 Arabidopsis systems

Arabidopsis thaliana has long been used as a model plant for molecular studies and *Arabidopsis* somatic embryogenesis systems have been well characterised. These systems include indirect and direct somatic embryogenesis. Somatic embryos can be obtained indirectly via callus from seedling (Huang and Yeoman, 1984), root protoplasts (Grady, 1990), immature zytotic embryos (Wu *et al.*, 1992; Luo and Koop, 1997) and leaf protoplasts (Luo and Koop, 1997), or directly from mesophyll protoplasts (O'Neill and Mathias *et al.*, 1993). But there are fewer studies on somatic embryogenesis using *Arabidopsis* compared with those using carrot or alfalfa systems.

1.4.2.4 Alfalfa microcallus suspension (MCS)

Callus cultures from various *Medicago* genotypes offer excellent experimental material for the analysis of molecular changes, especially during the early inductive phase. Treatment of dedifferentiated cells, grown in the presence of a weak auxin such as naphthalene acetic acid (NAA), with a strong auxin (2,4,-D) for a few minutes up to a few hours is sufficient to initiate organised growth in multicellular structures of MCS and to induce subsequent formation of embryos in hormone-free culture medium (Figure 1.1). This system also provides evidence for the key role of 2,4-D in the formation of embryogenic somatic cells. In addition, the use of these cultures allows the exact timing of the inductive phase and embryogenic induction to be controlled (Dudits *et al.*, 1991, 1995).

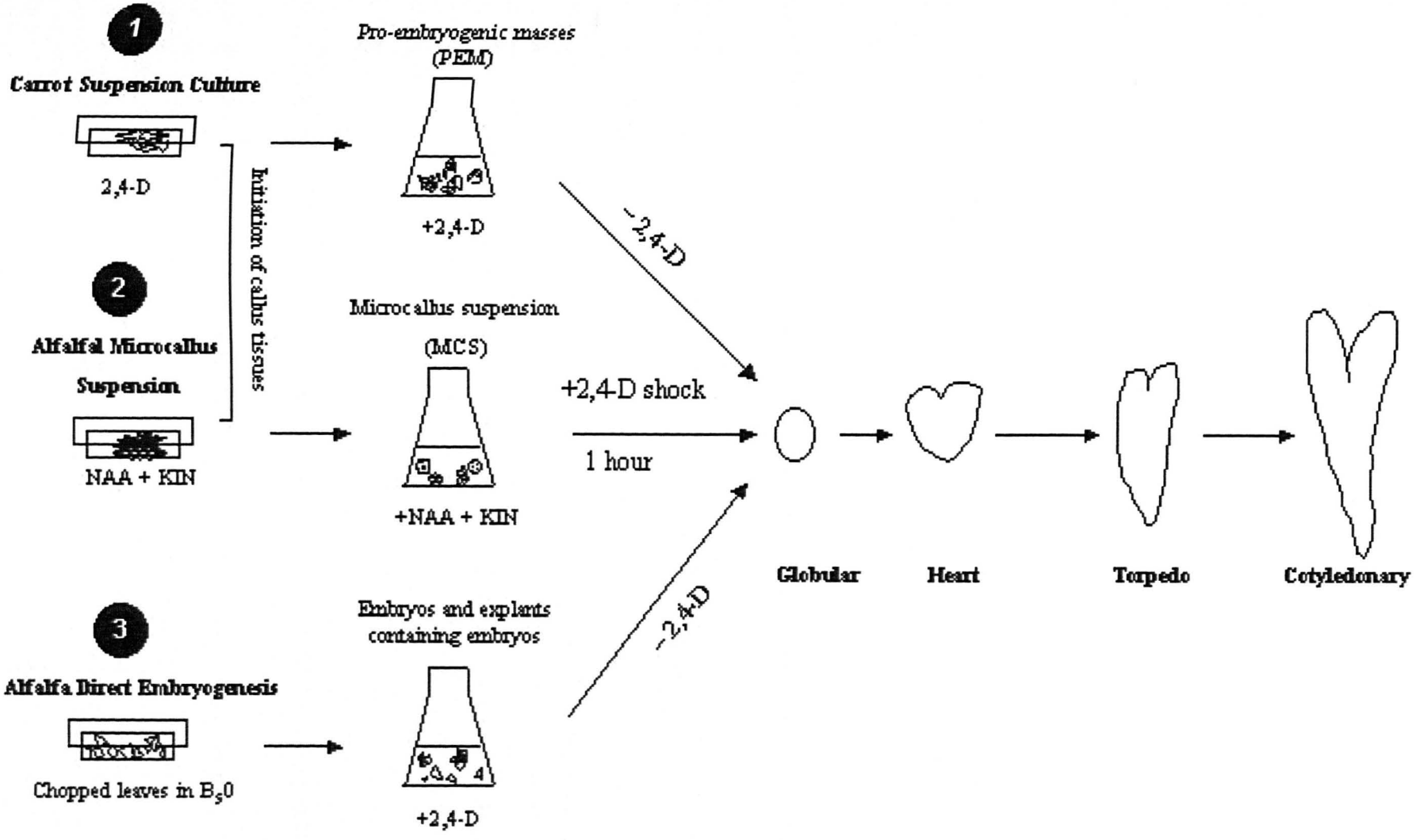


Figure 1.1 Key steps in initiation of embryogenic potential and embryo development in carrot and alfalfa cultures. 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, naphalene acetic acid; KIN, kinetin.

1.4.2.5 Alfalfa mesophyll protoplast cultures

Alfalfa (*Medicago sativa*) also offers a uniform single cell system for analyzing the dramatic developmental switch from somatic to embryogenic states under *in vitro* conditions (Dudits *et al.*, 1993, 1995). If leaf tissues are treated with cell wall-degrading enzymes, protoplasts can be released from fully differentiated mesophyll cells. In protoplast culture medium in the presence of auxin (2,4-D) and cytokinin (zeatin riboside) re-synthesis of the cell wall is followed by initiation of cell division and embryos can be formed from single cells without the intervening stage of callus formation (Kao and Michayluk, 1980; Song *et al.*, 1990). The frequency of this direct embryo formation can be increased by exposing the protoplast-derived cells to low voltage electrical fields (Dijak *et al.*, 1986).

1.4.2.6 The alfalfa explant direct somatic embryogenesis system

A highly efficient liquid medium system for alfalfa (*Medicago falcata*) direct somatic embryogenesis has been developed that exhibits regeneration from leaves (Denchev *et al.*, 1991a), petioles (Denchev *et al.*, 1990) and cotyledons (Denchev *et al.*, 1991b) (Figure 1.1). In this system, the embryogenic response relies on the ability of the differentiated cells in the primary explants to form embryos directly after wounding and application of auxin (2,4-D). As genotype is one of the most important factors affecting the embryogenic capacity in alfalfa (Brown and Atanassov, 1985), each embryogenic line has shown specific variations in terms of following the inductive treatment, the frequency of embryo formation and the synchrony of embryo development. Based on these characteristics, the embryogenic lines were classified as slow, middle and fast embryogenic genotypes. Experimental variation of induction conditions has shown that cells from a different part of a single leaf explant may have different requirements for the duration of the 2,4-D treatment required for embryo formation (Denchev *et al.*, 1991b). Never the less the system for direct somatic embryogenesis developed by Denchev and co-workers provides a useful experimental model for the molecular characterisation of embryogenic competence in differential cells and it has a series of advantages:

1. Direct somatic embryogenesis proceeds from cells within the leaf explants and this permits monitoring of the embryo induction process in mature tissues.
2. Embryogenic competence in the leaf tissue is limited by the specific physiological state of the explant and this state has been characterised in terms of explant age, endogenous phytohormone levels and *in vitro* tissue culture conditions.
3. Since phytohormones exert large physiological effects on plant cells, the prolonged cultivation of the embryogenic callus or cell suspensions in the presence of auxins makes it difficult to interpret the molecular changes leading to embryo determination. Moreover, in most of the indirect systems the embryogenic programme is already established in the PEMs. In this respect the direct embryogenic system allows molecular change detected during embryo induction to be directly related to the acquisition of embryogenic competence, induction and differentiation (Russinova, 1996).

1.5 Factors affecting the transition from somatic to embryogenic cells

1.5.1 Genotypes and source of explants

Genotype, type of explants and developmental stage seem to be the most important factors that determine the acquisition of competence of the plant explant cells to respond to an inductive signal (Yeung, 1995; Merkle *et al.*, 1990).

Somatic embryogenesis can be induced in cultures of various explant types: seedling and their fragments, petioles, leaves, roots, shoot meristems, seeds, cotyledons, and zygotic embryos. Immature zygotic embryos present the most frequently applied source of embryogenic cells and permit the induction of somatic embryogenesis in plant species which have been considered to be recalcitrant, such as grasses (Ahloowalia, 1991), conifers (Arnold *et al.*, 1996) and many dicotyledons (Raemakers *et al.*, 1995).

It has often been observed that the developmental stage of the explant is of prime importance for the transition of somatic cells into embryogenic cells (Ammirato, 1983; Conger *et al.*, 1983; Wernicke and Brettell, 1980; Tisserat *et al.*, 1979; Hartweck *et al.*, 1988; Pretova and Williams, 1986; Young *et al.*, 1987; Ghazi *et al.*, 1986; Ranch *et al.*,

1985; Barwale *et al.*, 1986; Karunaratne *et al.*, 1991; Vasil, 1987; Williams *et al.*, 1990; Wernicke and Milkovits, 1984, 1987; Trigiano *et al.*, 1989; Wenzler and Meins, 1985; Vasil, 1981; Linacero and Vazquez, 1986; Joarder *et al.*, 1986; Dolezelova *et al.*, 1992). The induced development pathway often reflects a "memory" of pathways either previously pursued, or about to be pursued (Carman, 1990). The physiological and morphologically maturity of tissue partially determines the time of occurrence and type of response possible in culture (Williams and Maheswaran, 1986). It has been proposed that the stage of embryogenic competence overlaps with the early stages of cell differentiation and specialisation. Thus, cells pass through a "window of response" in which they acquire embryogenic competence as they mature and differentiate. Within the heterogeneous population of cells in a more mature tissue, the embryogenically competent cells will be those which differentiate more slowly.

Genotype can play a major role in the embryogenic response of cultures in many species. The ability to undergo *in vitro* embryogenesis is conditioned by nuclear genes (Larsen *et al.*, 1991; Sonnino *et al.*, 1989). Individual genotypes within a given species vary greatly in embryogenic capacity (reviewed by Parrott *et al.*, 1991). For example, there are "embryogenic" and "non-embryogenic" lines of alfalfa (*Medicago falcata*) which vary considerably with respect to the proportion of competent cells within comparable explants (Denchev *et al.*, 1991b). Studies of the alfalfa system have led to the proposal that a distinct, potentially embrygenic population of somatic cells exists in all explants regardless of genotype. However, the process is limited by the specific physiological state of the explant (degree of tissue differentiation, phytohormone balance, culture conditions, etc.). Thus, differences in embryogenic competence between embryogenic and non-embryogenic alfalfa (*Medicago falcata*) reflect differences in the physiological status of the two lines and the breadth of the "window of response".

1.5.2 Stress factors

Somatic cells can acquire embryogenic potential as a result of different external chemical and physical stimuli, generally called stress factors. Stress is commonly thought of as an essential component of embryogenesis induced in microspore culture, i.e. androgenesis

(Touraev *et al.*, 1996; Dunwell, 1996). A positive influence of stress was also observed in cultures of somatic tissue where development of somatic embryos was induced (Dudits *et al.*, 1995). Embryogenic competence of *in vitro* cultured somatic cells can be stimulated by various factors, such as osmotic pressure, chlorides of heavy metals, pH, low or high temperature, starvation, mechanical wounding of explants or high auxin level (Kiyosue *et al.*, 1993). Osmotic pressure applied in an induction medium was reported to stimulate somatic embryogenesis in explants of *Panax. ginseng* (Choi *et al.*, 1998b) and *C. sinensis* (Akula *et al.*, 2000). Stress by starvation resulted in enhanced production of somatic embryos of *Daucus carota* (Lee *et al.*, 2001), while plasmolysing pre-treatment of *Panax. ginseng* explants was found to be an efficient method for increasing the efficiency of direct somatic embryogenesis (Choi *et al.*, 1999, 2001). In embryogenic cotton culture, a decrease in available nutrients and water created by the application of filter paper can significantly improve somatic embryo production (Kumria *et al.*, 2003). Antimicrotubule e.g. oryzalin treatment stimulated somatic embryogenesis in zygotic embryo cultures of *Ilex paraguariensis* (Rey *et al.*, 2002). Application of mild stress imposed by Fe^{2+} to protoplast cultures of alfalfa (*Medicago sativa*) induced an embryogenic transition in cells cultured in medium with non-embryogenic 2,4-D concentrations (Pasternak *et al.*, 2002). Cold-treatment combined with a proper Ca^{2+} level in the medium enhanced somatic embryogenesis in cell suspension cultures of *Astragalus adsurgens* (Luo *et al.*, 2003). Several stress factors (osmotic, heavy metal ion and dehydration stress) used in a carrot system were found to be crucial for induction of somatic embryogenesis in seedlings and flowers bud explants of *A. thaliana* (Ikeda-Iwai *et al.*, 2003).

All of the stress factors described induce a common reaction of somatic cells manifested by their de- and re-differentiation to somatic embryos. However, the molecular mechanism of the stimulatory effect of stress treatment on cell differentiation and morphogenesis remain to be revealed. The induction of abnormal cell division (MacDonald and Aslam, 1986; Zaki and Dickinson, 1995), accumulation of endogenous auxins (Zimmerman, 1993), changes in the cytoskeleton components (Iqbal *et al.*, 1994), or the induction of stress proteins (Rey, *et al.*, 2002; Puigderrajols *et al.*, 2002), have been proposed to explain the mode of stress factor activity.

One hypothesis of the mechanisms involved in stress-induced embryogenesis highlights the importance of the interaction between auxin and stress signaling which results in acquisition of embryogenic competence of somatic cell, by broad cellular reprogramming manifested at different levels (Feher *et al.*, 2003). Whatever the mechanism, stress treatment triggers expression of factor(s) that affect gene expression and cell cycle regulation and thus induce somatic embryogensis (Gaj, 2004 in press).

1.5.3 Plant growth regulators

As regulators of developmental switches, several hormones have been implicated in the control of somatic embryogenesis. Auxins and cytokinins are the main growth regulators in plants involved in the regulation of cell division and differentiation. The influences of exogenously applied auxins, particularly 2,4-dichlorophenoxyacetic acid (2,4-D), on the induction of somatic embryogenesis are well documented. They have been identified as one of the key inducers of embryogenic development in somatic plant cells cultured in vitro (for reviews e.g., Dudits *et al.*, 1991; Yeung, 1995). The minimum concentration or the duration of 2,4-D treatment required for this inductive effect differs between species and genotypes (Dudits *et al.*, 1991; Zimmerman, 1993) and between explants (Zimmerman, 1993). For instance, carrot petiole explants (Ammirato, 1985), hypocotyl explants (Kamada and Harada, 1979), and single cells isolated from established suspension cultures (Nomura and Komamine, 1985) require exposure to exogenous auxin for 1, 2 or 7 days, respectively, before they are competent to undergo embryogenesis upon auxin removal. Microcallus cells of alfalfa (*Medicago sativa*) require only a short (a few minutes to a few hours) pulse of auxin before they are competent to initiate embryogenesis in plant growth regulator-free medium (Dudits *et al.*, 1993). Very high concentrations of exogenous auxins were required for the induction of somatic embryogenesis in some plant systems e.g. in cultures of cotyledons of *Serenoa repens* or *Pisum sativum* where 452 μM 2,4-D or over 200 μM of NAA were found to be effective (Gallo-Meagher and Green, 2002; Özcan *et al.*, 1993). Furthermore, structural studies revealed that only a few cells in the inoculated primary explant appeared to be competent for embryogenic induction (Schmidt *et al.*, 1997). All of these observation showed that differences can originate from different degrees of sensitivity

of cells or tissues toward auxins (Dudits *et al.*, 1995). The possible relationship between auxin sensitivity and embryogenic potential was indicated by comparative studies on embryogenic (A2) and non-embryogenic (R15) alfalfa (*Medicago sativa*) genotypes (Bogre *et al.*, 1990). The proposed significance of auxin sensitivity in somatic embryogenesis might help to explain differences between plant species, genotypes or cells in the same explants, or in explants with different origins in their capability to become embryogenic (Dudits *et al.*, 1995).

Auxins not only stimulate cell division, but also disrupt the connection between adhering cells by loosening hemicelluloses from the cellulose microfibrils (Hayashi, 1991) and promote cell elongation by vacuole expansion and formation of microtubules transverse to the elongation direction (Shibaoka, 1991). So long as auxin treatment does not eliminate highly cytoplasmic cells whose daughter cells adhere to each other to form multicellular structures, embryo development is possible. If the auxin concentration is too high or subculturing is performed too often, the small and highly cytoplasmic, "embryogenic", cells disappear from the culture, because further disruption and elongation of cells takes place (Emons, 1994). In some systems, cytokinins, instead of auxins, were found to be effective in somatic embryogenesis induction (Asano *et al.*, 1996; Castillo and Smith 1997; Carimi *et al.*, 1999; Chen and Chang, 2001; Nanda and Rout, 2003), but the cytokinin-induced model of embrygenesis is generally rare.

1.5.4 pH

Numerous studies have demonstrated that changes in cytoplasmic pH (pHc) occurred during metabolic and developmental transitions in a large variety of cells, including plant cells (for review, Frelin *et al.*, 1988; as a review, Kurkdjian and Guern, 1989; Pichon and Desbiez, 1994; Bibikova *et al.*, 1998). Pichon and Desbiz (1994) found that cytoplasmic pH in *Bidens pilosa* was correlated with cell division. Alkalinisation promoted the cell cycle in the meristematic region of the hypocotyls, while acidification inhibited it. Initiation of root hair cells in *Arabidopsis* could also be characterised by the acidification of the apoplast and the alkalinisation of the cytoplasm (Bibikova *et al.*, 1998).

Characteristic changes in intracellular pH are hypothesised to be associated with the transition from the somatic to the embryogenic cell state. A relationship between medium (and cellular) pH and developmental state has been suggested by experiments with wounded carrot zygotic embryos cultured in the presence of 1 μM NH_4Cl (Smith and Krikorian, 1990a, b). NH_4Cl-induced cellular alkalinisation allowed the establishment of a continuous culture of pre-globular stage pro-embryos. Medium pH was decreased in these cultures (down to pH 4), which was correlated with increased pHc (cytoplasmic pH). The development of embryos could only be advanced if the medium pH was raised to approximately pH 5.7. Cells of an alfalfa embryogenic-type callus have been found to have higher average pHc values compared to cells from a non-embryogenic type (Schaefer, 1985). In alfalfa leaf protoplast-derived cells, cytoplasmic and vacuolar alkalinisation and medium acidification were shown to be correlated with the activation of cell division (Pasternak *et al.*, 2002). Small, cytoplasm-rich embryogenic cells had a tendency to exhibit higher vacuolar pH values in comparison to the non-embryogenic vacuolated cells (Pasternak *et al.*, 2002). It has been proposed (Fehér *et al.*, 2003) that the large difference between the vacuolar pH of the embryogenic and non-embryogenic cell types is related to differences in vacuolar functions (for reviews, Wink, 1993; Marty, 1999; Ratajczak, 2000). Elongated, differentiated cells have large, central, lytic vacuoles with more acidic pH, while the small, dedifferentiated cells have several small storage-type vacuoles (Fehér *et al.*, 2003).
In the same leaf protoplast system, buffering of the medium pH with 10 mM MES prevented embryogenic cell formation under inductive conditions. Increased transport across cell membranes, inhibited by extracellular MES, is possibly an important process in the metabolic reprogramming of embryogenic cells (Pasternak *et al.*, 2002).

1.5.5 Ca^{2+}

Ca^{2+} functions as a key regulator of many cellular and physiological events in plants (for review, Sanders *et al.*, 1999). Current models of Ca^{2+} -mediated signaling emphasise the significance of a transient change, usually an increase, in cytoplasmic calcium concentration, followed by the perception of such changes by calcium-binding proteins. The involvement of Ca^{2+} in a wide variety of stimulus-response pathways in plant cells raises several

questions concerning how the same messenger can regulate many different responses. The amplitude, duration, frequency and location of the Ca^{2+} signal can be considered as key features in the determination of different messages (Fehér *et al.*, 2003).

The increase in intracellular Ca^{2+} concentration after fertilisation of egg cells has been demonstrated in both animal and plant cells (Stricker, 1999; Antoine *et al.*, 2000). The evidence from brown algae (Robinson *et al.*, 1999) and flowering plants (Antoine *et al.*, 2000) suggested a hypothesis that gamete fusion-induced calcium influx plays a direct role in egg cell activation (Fehér *et al.*, 2003).

The dependence of somatic embryogenesis on external and internal calcium concentrations has been demonstrated in different systems. In an embryogenic carrot cell suspension, an upward shift in the exogenous calcium concentration occurred at the time of transfer to auxin-free embryo differentiation medium increased the numbers of somatic embryos approximately 2-fold (Jansen *et al.*, 1990). The elevated calcium concentration counteracted the inhibitory effect of 2,4-D on embryo development (Jansen *et al.*, 1990). An exogenous Ca^{2+} concentration higher than 200 μM is required for optimal embryo formation (Overvoorde and Grimes, 1994). The application of either Ca^{2+}-channel blockers or the Ca^{2+} ionophore A23187 inhibited embryo initiation. These data indicate that exogenous Ca^{2+} and the maintenance of cellular Ca^{2+} gradients are required for proper embryo development *in vitro* (Fehér *et al.*, 2003).

Additional data support the hypothesis that Ca^{2+}-dependent protein kinases (CDPKs) are involved in signaling pathways involved in the formation of somatic embryos. In sandalwood, two CDPKs could be detected in protein extracts of embryogenic cell cultures (Steenhoudt and Vanderleyden, 2000). Their strong Ca^{2+}-dependent activities were detected in PEMs as well as in somatic embryos of different stages, but not in regenerated plantlets (Jackson and Casanova, 2000). The expression of the MsCDPK3-encoding gene has been shown to increase during the early phase of 2,4-D-induced embryogensis from cultured alfalfa cells (Davletova *et al.*, 2001).

1.6 Structural characteristics of embryogenic cells

Although there are some problems associated with the recognition and characterisation of embryogenic cells in somatic cell cultures (Street and Withers, 1994; Yeung, 1995; Schmidt *et al.*, 1997), embryogenic cells in a number of systems share similar structural features. They are small, highly cytoplasmic, and often have an accumulation of starch within the plastids (Yeung, 1995; Emons, 1994). In carrot cell suspension cultures, cells able to undergo embryogenic development pathway generally appear as pro-embryogenic masses (PEMs) composed of dense cytoplasmic small cells (Halperin, 1966; Halperin and Henson, 1967). These cells are smaller than other cells in the culture, with thin walls, and they contain numerous small starch grains. The cells are highly cytoplasmic with numberous organelles, and are capable of repeated cell division.

Formation of embryogenic cells can be correlated with characteristic morphological changes. Toonen *et al.* (1994) revealed the fate of embryogenic carrot cells by video cell tracking. The single cell fraction (<22 μm) of the established embryogenic cell culture contained cells that could be classified into five morphological groups. Although all cell types were capable of developing into somatic embryos with varying efficiency, the highest frequency was observed in the case of small, spherical, cytoplasm-rich cells.

Nomura and Komamine (1985) reported that after density gradient centrifugation, a fraction of small, isodiametric, cytoplasm-rich cells that could initiate embryo formation with 90% frequency could be isolated. This cell type was designated as State 0, or embryogenic competent cells which, in the presence of auxin (2,4-D), formed the State 1 embryogenic cell clusters, consisting of less than 10 cytoplasm-rich cells (Nomura and Komamine, 1995). These clusters differentiated into globular embryos on auxin-free medium. In contrast, the observation from the cell cultures initiated from petioles of *Medicago sativa* has shown that single cells were unable to develop into somatic embryos and the fraction trapped between 224 and 500- μm mesh represented embryogenic cell clusters consisting of small, rapidly dividing cells (Xu and Bewley, 1992). Similar observations have been made in the case of *Picea abies* (Filonova *et al.*, 2000).

In alfalfa, leaf protoplast-derived cells cultured at different 2,4-D concentration can develop into either embryogenic or non-embryogenic cell types. Similar morphological traits could be recognised when protoplasts of embryogenic and non-embryogenic genotypes were cultured at the same concentration. In both cases, embryogenic cells were small, spherical

and densely cytoplasmic, while non-embryogenic ones were elongated and highly vacuolated (Bögre *et al.*, 1990; Dudits *et al.*, 1991; Fehér *et al.*, 2002; Pasternak *et al.*, 2002). Embryogenesis competent cells formed in chicory leaf explants were also characterised by dense cytoplasms (Blervacq *et al.*, 1995).

However, by using time-lapse photography, Backs-Husemann and Reinert (1970) described an elongated single vacuolated cell able to develop into a somatic embryo in suspension culture. Guzzo *et al.*, (1994; 1995) and Schmidt *et al.*, (1997) also showed that in carrot explants, a small subset of a particular type of elongated single cells are the first to acquire the competence to form embryogenic cells, rather than the small, isodiametric and cytoplasm-rich cells which are generally believed to contain a sub-population of embryogenic competent cells.

Several reasons make it difficult to identify competent cells for embryogenesis. First, as described above, recording of the developmental fate of all types of carrot cells from established embryogenic suspension cultures by cell tracking revealed that competent cells have a highly variable appearance that prevents their identification on the basis of morphology (Toonen *et al.*, 1994). The second is the fact that only a limited number of cells actually undergo the transition of somatic into embryogenic cells. Except for the ability to form embryogenic cells, these cells are indistinguishable from the majority of analogous cells (Schmidt *et al.*, 1997; Yeung, 1995). Finally, the cellular changes observed in somatic explant cells that have responded to the inducing treatment in general have not been proved to be essential to the formation of embryogenic cells. Further ultrastructural characterisation may provide a structural basis for the determination (Yeung, 1995). The cytological characteristics of the induced cells are indicative of high metabolic activity, supporting a fast rate of cell division and growth, e.g. frequency of polysomes, rise in endomembranes, active dictyosomes. The nuclear features (envelope invaginations and the close proximity to mitochondria and plastids) indicate increased interaction between cytoplasm and organelles. The storage of starch in plastids is considered to be a defining characteristic of embryogenic competence (Karlsson and Vasil, 1986; Profumo *et al.*, 1987; Bonnelle *et al.*, 1990; Fransz and Schel, 1991).

Judging from the ultrastructural studies, embryogenic cells are metabolically active. The abundance of organelles is an indicator of rapid cell growth (Tautorus *et al.*, 1991). Thus, mitogenic activity is an essential property of embryogenic cells.

1.7 Chromatin remodeling

Chromatin structure, the DNA organisation around basic nuclear proteins (histones), is intrinsically involved in the regulation of nuclear processes, such as DNA repair, replication and especially transcription (Varga-Weisz and Becker, 1998). Chromatin structure changes in a dynamic way and is continuously remodeled during development. Chromatin-dependent gene silencing is a common mechanism for maintaining the differentiated state of cells. Thus chromatin remodeling is necessarily linked with cellular dedifferentiation and the switching of cell fate (Fehér *et al.*, 2003).

It can be hypothesised that chromatin remodeling plays two major roles during the early stages of somatic embryogenesis. Dedifferentiation requires unfolding of the supercoiled chromatin structure, in order to allow the expression of genes inactivated by heterochromatin formation during differentiation, and subsequent chromatin remodeling can result in the specific activation of a set of gene required for embryogenic development (Fehér *et al.*, 2003).

1.8 Secreted proteins and the embryogenic cell

During the last few years, an increasing body of experimental data has accumulated on the role of extracellular proteins on somatic embryo induction and development. Callose deposition in walls of embryogenic cells and the formation of a glycoproteic extracellular matrix around superficial cells of globular embryos can serve as cytological markers of cell reactivation and embryogenic differentiation (Dudits *et al.*, 1990, 1991; Pedroso and Pais, 1995). Embryogenic competent leaf cells in chicory had thicker walls, exhibiting a brighter autofluorescence of cellulose under UV light, compared to non-reactivated mesophyll cells (Blervacq *et al.*, 1995). In direct embryo formation from leaf explants of *Camellia japonica*, as an early marker of the morphogenetic response, cell walls

underwent characteristic changes, including the deposition of callose on the surfaces of induced cells (Pedroso and Pais, 1995). This was followed by the deposition of other materials (cutin) characteristic of somatic embryogenesis, with lipid transfer proteins being presumed to be involved in this process. Expression of lipid transfer proteins is a well-known early marker of somatic embryo induction in different systems (Sterk *et al.*, 1991; Schmidt *et al.*, 1997; Sabala *et al.*, 2000), as it is linked to the formation of the protoderm layer in developing somatic and zygotic embryos (Thoma *et al.*, 1994; Vroemen *et al.*, 1996; Toonen *et al.*, 1997a).

Arabinogalactan proteins (AGPs) are a class of proteoglycans with poly- and oligosaccharide units covalently attached to their protein moiety (Van Holst and Klis, 1981), that form a complex with a specific synthetic β-glucosyl Yariv reagent (βGlcY) (Yariv *et al.*, 1962). In plants, a wide variety of AGPs are expressed and implicated in diverse processes of plant growth and development (for review, Showalter, 2001), including somatic embryogenesis (for review, Vroemen *et al.*, 1999). Although their exact functions are not clear, AGPs are presumably involved in molecular interactions and cellular signaling at the cell surface. Their role during somatic embryo formation was demonstrated by the addition of the β-D-glucosyl Yariv reagent (βGlcY), which interacts with AGPs, to the culture medium. Different AGP populations are able to increase or decrease embryogenic potential in carrot suspension cultures. In old cell lines that had lost the ability to develop somatic embryos, embryo formation could be re-initiated by addition of carrot-seed AGPs (Kreuger and Van Holst 1993). Addition of Norway spruce-seed AGPs to an immature type B spruce somatic embryo culture resulted in the formation of more-developed embryos. But to complete development into the mature type A somatic embryos, addition of total seed extract was required, indicating that besides AGPs other components are required (Egertsdotter and Von Arnold 1995). The ratio of different AGPs in suspension cultures can determine the embryogenic potential. The AGP fraction isolated with the monoclonal antibody ZUM18 (ZUM18AGPs) was shown to increase the percentage of embryogenic cells in carrot suspension cultures about twofold. An approximate two fold decrease in the percentage of embryogenic cells was shown by the AGP fraction isolated with the ZUM15 antibody (ZUM15AGPs). The effect of ZUM15AGPs was similar to AGPs isolated from non-embryogenic suspension cultures;

ZUM18AGPs as well as ZUM15AGPs are present in seeds and in suspension cultures (Kreuger and Van Holst, 1995).

The first observation of the *ts11* temperature sensitive carrot mutant indicated that extracellular, secreted proteins may have important roles during somatic embryo development. *ts11* somatic embryos were arrested at the globular stage with aberrant protoderm formation at elevated temperature (32°C) (de Jong *et al.*, 1992). However, the developmental arrest at elevated temperatures could be bypassed by the addition of culture medium in which fully embryogenic lines had been grown. The secreted molecule was identified as a 32-kDa protein with homology to an endochitinase (de Jong *et al.*, 1992). In embryogenic cultures of *Cichorium*, the major secreted proteins were also identified as chitinases, glucanases and an osmotin-like protein, all of which accumulated at a significantly higher level in embryogenic compared to non-embryogenic cultures (Helleboid *et al.*, 2000b). Similarly, in embryogenic alfalfa cultures, alterations in the levels of extracellular proteins homologous to the carrot endochitinases have been reported following the removal of 2,4-D (Poulsen *et al.*, 1996). Carrot AGPs contained glucosamine and *N*-acetylglucosamine that were sensitive to endochitinase cleavage (van Hengel *et al.*, 2001). The embryogenic competence of protoplasts could be restored and enhanced by the application of AGPs, endochitinase-cleaved forms of which were more efficient (van Hengel *et al.*, 2001). It was hypothesised that chitinase-modified AGPs are extracelluluar molecules capable of controlling or maintaining the embryogenic competent cell state (van Hengel *et al.*, 2001).

1.9 Polarity establishment and embryo formation

In seed plants, the egg cell and zygote exhibit apical-basal polarity (Russell, 1993; Vroemen *et al.*, 1996, 1999; Scheres and Benfey, 1999). Polarised development of the zygote and the embryo includes the formation of shoot and root meristems, which maintain the apical-basal polarity throughout plant development (Jurgens, 2001). These meristems comprise groups of undifferentiated cells and serve as cellular sources for tissue and organ differentiation.

The initial zygotic division in higher plants is asymmetric (Scheres and Benfey, 1999). This event establishes the basic polarity of the plant which probably determines subsequent pattern formation (Jurgens *et al.*, 1997). In *Arabidopsis*, embryonic cell divisions are very regular and the *gnom* mutation, which affects the first asymmetric division, causes abnormal pattern formation (Busch *et al.*, 1996).

The establishment of polarity is probably an important event, not only for zygotic, but for somatic embryogenesis as well. In alfalfa, where leaf protoplasts can be used to induce direct development of embryogenic cells (Dijak and Simmonds, 1988; Song *et al.*, 1990; Dudits *et al.*, 1991), 2,4-D stimulates the formation of asymmetrically dividing cells, and the type of division determines further development. Nomura and Komamine (1985) showed that isolated small, cytoplasm-rich carrot cells, after unequal first division and polarised synthesis of macromolecules, have the ability to develop into somatic embryos. Even if morphological asymmetry is not obvious, the unequal distribution of cellular determinants can be decisive in the determination of cell developmental pathways following division (Fehér *et al.*, 2003). The polarity of the egg cell is evident from the position of the nucleus at the cytoplasm-rich chalazal pole, while the micropylar pole is highly vacuolated (Russell, 1993). The microtubular cytoskeleton is particularly dense near the nucleus and has no specific orientation. In alfalfa, protoplast-derived embryogenic cells were also characterised by disordered microtubles when compared to non-embryogenic control cells (Dijak and Simmonds, 1988).

Physical, electrical, ionic, and hormonal signals have been implicated as important influences in the establishment of polarity and plant morphogenesis (Wardlaw, 1968; Schnep, 1986; Osborne, 1989). In carrot, electrical polarity was found to be established in clusters undergoing apparently disorganised proliferation in the presence of 2,4-D. The electrical polarity was similar to that found in organised somatic embryos (Nomura and Komamine, 1995). This observation suggested that the potential to undergo embryogenesis was present even before 2,4-D was removed (Gorst *et al.*, 1987). Dijak *et al.* (1986) observed that the application of a low voltage electrical field greatly enhances the formation of somatic embryos from alfalfa. An alternative means of imposing polarity is the attachment of cells to a substrate (Merkle *et al.*, 1990). The production of embryos of sweet potato (*Ipomoea batatas*) from liquid culture was improved by anchoring pre-

embryogenic aggregates to alginate beads to maintain a physiological polarity (Chee and Cantliffe, 1989). This mild electrical treatment may have perturbed the organisation of the cell leading to the establishment of polarity and organised development (Yeung, 1995). Calcium ions can mediate a large number of developmental processes in the plant. Unequal distribution of Ca^{2+} channels and the established Ca^{2+} gradients are also important in early determination of the axis (Pu and Robinson, 1998). A calcium influx was triggered in the vicinity of the sperm entry site and subsequently spread to the whole egg cell during the *in vitro* fertilisation of maize (Antoine *et al.*, 2000). In sunflower protoplast-derived cells, a translocation of Ca^{2+} channels depended on the division type was detected by the fluorescent probe DMBodipy-PAA (Vallee, 1997; Xu *et al.*, 1999). There is evidence that the polar localisation of F-actin filaments and the polarised secretion of cell wall material play roles in axis fixation (Fowler and Quatrano, 1997; Belanger and Quatrano, 2000; Vroemen *et al.*, 1999).The role of polarised secretion in the disruption of this process was indicated by the results of Brefeldin A treatment, an inhibitor of Golgi function (Capitanio *et al.*, 1997). Auxin transported in a polar manner along the shoot-root axis requires efflux carriers such as PIN1 (for review, Palme and Gälweiler, 1999). BrefeldinA was shown to abolish the polar localisation of PIN1 and, consequently, the polar transport of auxin. The role of polarised auxin transport was also implicated in the establishment of apical-basal patterning in *Fucus* embryos (Basu *et al.*, 2002).

1.10 Auxin as a positional and a patterning signal molecule

The results presented so far implicate auxin as a key signal molecule in providing positional information within the apical region of the embryo for the co-ordination of correct cellular patterning, particularly during the transition period from globular to heart stage. Auxin has proved a difficult molecule to localise in tissues due to its highly diffusible character and occurrence in both active and inactive (conjugated) forms (Normanly and Bartel, 1999). Shoot meristems and leaf primordia are regarded as the main sites of synthesis, with the polar auxin transport system holding the key to many responses. Vascular tissue formation follows the flow of auxin (Aloni, 1987; Mattsson *et*

al., 1999), which is canalised into files of cells so that connected vascular strands form (Sachs, 1991). Auxin controls much of post-embryonic development, especially plant architecture, through the modulation of meristem activity and cell expansion in response to environment factors (Hobbie, 1998).

Auxin transport is therefore a key to understanding much of the role of auxin within the plant. The chemiosmotic theory proposes that auxin requires an influx and efflux carrier in order to move through cells and tissues. This requires anion symport (influx) and efflux carrier proteins. AUX1 is recognised as a candidate for the influx carrier (Bennett *et al.*, 1996), while the *PIN* gene family constitutes the putative transport protein of the efflux carrier complex.

For the efflux, its cellular localisation needs to be precise as it might be expected to determine the course of auxin flow (Souter and Lindsey, 2000). At present, seven *PIN* genes have been identified, whilst more than ten different *PIN* homologues have been found in *Arabidopsis*. *PIN* genes have also been identified in other species, such as maize, rice and poplar. The high degree of similarity between monocot and dicot *PIN* genes indicates a conserved function for PIN proteins throughout the plant kingdom (Souter and Lindsey, 2000). In *Arabidopsis* members of this family of transporters have different expression patterns within time and space, and so offer the plant a means by which auxin can be transported precisely. PIN1 has shown to be linked to the development of vascular tissue. PIN1 is located at the basal end of cells within the vascular stele (Gälweiler *et al.*, 1998). During embryogenesis, *PIN1* expresses at the mid-globular stage and becomes polarised, before the two cotyledons have started to develop. By the heart stage the expression pattern of PIN1 very much resembles the pattern it takes throughout the rest of the plant's post-embryonic development, forming a characteristic Y shape from the two cotyledons to the basal end of the embryo (Steinmann *et al.*, 1999).

Recent direct evidence for the existence of auxin gradients that correlate with a physiological response is revealed by the highly sensitive technique of GC-MS (Uggla *et al.*, 1996, 1998). The results showed that the presence of a steep radial gradient of auxin across the vascular cambium in *Pinus sylvestris* (L.). The gradient of auxin across the tissue appears to provide positional information for the developing tissue, with possibly other morphogen gradients or cell-cell communication systems determining the precise

cell division patterns and cell fates required to produce the specific cell types that exist within this tissue (Souter and Lindsey, 2000).

Lui *et al.* (1993) first reported the use of auxin transport inhibitors to study development in cultured zygotic embryos of *Brassica juncea* (Lui *et al.*, 1993). Their results showed that inhibition of auxin transport at the globular stage leads to the formation of embryos which lack bilateral symmetry at the heart stage. Bilateral symmetry is established when the two cotyledons form on either side of the shoot meristem region. Instead of two cotyledons, embryos developed with fused and collar-like cotyledons, which interestingly phenocopied the known auxin transport- defective pin1 (Okada *et al.*, 1991) and *gnom* mutants (Steinmann *et al.*, 1999).

Studies on the *POLARIS* gene of *Arabidopsis* provide further information on the role of auxin in defining position and cell activies during embryonic and seedling root development. The POLARIS gene promoter is up-regulated by auxin very rapidly, within minutes, and its spatial expression pattern represents a useful marker of auxin localisation in the root (Topping and Lindsey, 1997, and unpublished data). It was found that correct spatial patterning of *POLARIS* expression is disrupted significantly only in the most severe, ball-shaped *gnom* seedlings suggesting that these individuals, but not the more conical-shaped *gnom* seedlings, are defective in polar auxin transport (Topping and Lindsey, 1997). This is consistent with the observation of defective PIN localisation in *gnom* embryos (Steinmann *et al.*, 1999), and suggests that auxin provides a chemical framework for the patterning of apical-basal gene expression and cellular activity in both embryo and seedling.

1.11 Gene expression during somatic embryogenesis

The processes during which somatic cells acquire embryogenic competence obviously involve the reprogramming of gene expression patterns (Fehér *et al.*, 2003). During this progression, the morphology, physiology and metabolism of the cells are significantly altered due to dedifferentiation, activation of cell division and a change in cell fate. All of these changes are dependent on the inactivation of genes functioning in differentiated cells and the activation of those required for the above processes. Obviously, the overall

reprogramming of gene expression has to be governed by regulator genes, including those encoding transcription factors (Fehér *et al.*, 2003).

A variety of studies have indicated that the synthesis of embryogenic proteins is controlled at the level of gene transcription. There are perhaps as many as 20,000 – 30,000 genes expressed during embryogenesis (Dure, 1985; Goldberg *et al.*, 1989; Lindsey and Topping, 1993; Thoms, 1993) and many of them may comprise multigene families (Casey *et al.*, 1986). Most of them are "housekeeping" genes which influence the cellular responses that are components of normal metabolic functions such as cell division, stress response, respiration and cell wall synthesis (Dudits *et al.*, 1995; Racusen and Schiavone, 1990; Lindsey and Topping 1993). It is possible that activation of these genes may follow an unusual order or variation in the level of expression or in restricted region of cells that redirects cellular activities and, in turn, alter morphology. Lindsey and Topping (1993) and Goldberg *et al.*(1989) described the diverse patterns of mRNA abundance and proposed that classes of transcription can be recognised as being restricted to specific developmental stages of embryogenesis. In this respect, many of the genes expressed during embryogenesis would also be expected to be transcriptionally active in other metabolically active cells such as meristematic tissues (Lindsey and Topping, 1993; Dodeman *et al.*, 1997).

The generation and characterisation of mutants has been recognised as an important approach to identification of genes essential for embryogenesis, which is expected to be more fruitful in the future (reviewed by Lindsey and Topping 1993; Dodeman *et al.*, 1997; Goldberg *et al.*, 1989; Jurgens *et al.*, 1991; Aeschbacher and Benfey 1992; Meinke, 1991; Kaplan and Cooke, 1997). Using a chemical mutagenic strategy, Mayer *et al.* (1993) estimated that a relatively small number of genes, approximately 40 or 50 genes in total, may be sufficient to control pattern formation in the *Arabidopsis* embryo. This number is similar to the estimate for the control of patterning in the *Drosophila* embryo (St. Johnston and Nusslein-Volhard,1992). The use of new techniques, such as transposon tagging or promoter trapping followed by partial sequencing will allow identification of regulatory genes and their encoded products (Hofte *et al.*, 1993; Topping *et al.*, 1994; Dodeman *et al.*, 1997).

The inductive phase and progression of embryo development rely on various 'decision-making' molecular events during development (Dudits *et al.*, 1995). If certain gene products are not detected at other times in development, they may be defined as stage-specific and, as such, are valuable as diagnostic molecular markers of overall morphogenetic sequence (Racusen and Schiavone, 1990). Therefore isolation and characterisation of stage-specific marker genes will also be valuable for a comprehensive molecular analysis of this developmental pathway (Dudits *et al.*, 1995).

The most common approach to identify somatic embryogenesis-related genes is to compare gene expression in somatic embryos to that of non-embryogenic cells. This approach has resulted in the identification of a few abundant transcripts linked to specific stages of embryogenesis, rather than to the induction period of embryogenic development (for review, Zimmerman, 1993). In carrot, Lin *et al.* (1996) used an approach whereby the population of globular embryos was compared to that of seedlings. The sensitivity of the cDNA library screening was improved by using a subtracted probe enriched for globular embryo enhanced transcripts. Thirty-eight cDNA clones were identified to represent genes with altered expression during somatic embryogenesis. The majority of proteins encoded by these genes could be classified into the categories of cell wall proteins, enzymes, pathogenesis-related (PR) proteins, heat-shock proteins, late-embryogenic abundant (Lea) proteins, oleosins, a globular-like protein, histones, ribosomal proteins, elongation factor 1 α and ubiquitin fusion protein. The expression patterns of these clones were highly variable and could be considered as markers of the developmental stages of embryos (Fehér *et al.*, 2003).

Direct somatic embryogenesis is well established for various alfalfa species and genotypes. More advantageously, there are also many closely related genotypes that are non-embryogenic under the same conditions. It is not surprising, therefore, that many attempts have been made to identify early embryogenesis-related genes in these species (Fehér *et al.*, 2003). Giroux and Pauls (1997) compared mRNA populations of established cultures of embryogenic and non-embryogenic *Medicago sativa* L. genotypes using a conventional differential cDNA library screening approach. The screening resulted in three cDNA clones (ASET1-3) specifically present in cells of the embryogenic genotype. Analysis of these clones suggested that their encoding proteins could be important in membrane

related signaling events. In an alternative approach, cDNAs coding for small heat shock proteins (Dudits *et al.*, 1991), a proline-rich protein (Györgyey *et al.*, 1997) and a calcium-binding protein with unknown function (Dudits *et al.*, 1991) were isolated by differential screening of a cDNA library from embryogenic *Medicago sativa* cell cultures treated with a high 2,4-D concentration to induce somatic embryogenesis.

In *Medicago falcata*, somatic embryo development can be induced in leaf explants by parallel wounding and 2,4-D application (Denchev *et al.*, 1991). mRNA samples were isolated at different time points, following the induction of direct somatic embryogenesis, and converted to cDNA prior to RNA arbitrarily primed PCR (RAP-PCR) by Fowler *et al.*, (1998). Two different primer combinations were used and the cDNA fragments with differential accumulation during embryo induction were identified and sequenced. Most of the clones had no significant homology to database sequences and the expression patterns of only two of them have been verified. The clone A1.4 was characterised as a calnexin homologue with a potential chaperone function (Huang *et al.*, 1993). The clone A2.5 exhibited homology with a family of multidrug resistance genes, like the yeast *SNQ2* gene (Servos *et al.*, 1993). It was expressed only at the time of globular embryo formation.

In another approach, PCR-based cDNA subtraction was used to identify differentially expressed genes in induced and non-induced *Medicago falcata* leaves (Russinova *et al.*, 1998). Nearly 100 different clones were identified. The sequences obtained revealed the presence of many regulatory genes (such as genes of transcription factors, kinases, the phosphatase PP2C, and auxin-induced genes), several ribosomal proteins, translational and post-translational proteins, signal transduction components, cytoskeletal proteins, membrane transport proteins, wound and stress-related proteins and proteins and proteins involved in electron transport. However, many of the identified genes could be induced by wounding or 2,4-D alone. Thus, further analysis is required to elucidate the potential significance of the protein products of these genes during somatic embryogenesis.

1.11.1 Gene related to auxin (2,4-D) treatment

The resetting of the whole ontogenic program by initiation of somatic embryogenesis requires an essential reprogramming of the gene expression pattern. It is evident that auxin

(2,4-D) treatment can re-program differentiated somatic cells to become totipotent and to reach a developmental potential similar to that of the egg cells after fertilisation. The major signal transduction events involved in the triggering of the development by fertilisation in sea urchin embryos have been stressed by Epel (1990). According to this overview, the major changes can be characterised as membrane depolarisation, polyphosphoinositide hydrolysis, Ca^{2+} release, Na^{+}, H^{+} exchange, pH increase, elevated oxygen consumption and H_2O_2 production. Considerable similarity was found when comparing these early events in signal transduction and cellular responses between fertilised egg cells and auxin-treated somatic plant cells (reviewed by Davies, 1987). As shown by several lines of evidence, the primary mechanism of auxin action is related to the binding of the hormone to proteins (Dudits *et al.*, 1995). Results from several studies have clearly proved the presence of auxin-binding proteins or the existence of auxin receptors in the plasmalemma and in the ER (endoplasmic reticulum) (Dohrman *et al.*, 1978; Shimoura *et al.*, 1988; Barbier-Brygoo *et al.*, 1989; Inohara *et al.*, 1989). Despite the accumulating data about auxin-binding proteins, the direct or indirect involvement of these molecules in the mediation of auxin action has not yet been determined (Dudits *et al.*, 1995). Becraft (1998) has reviewed the roles of receptor kinases in plant development. In carrot, LoSchiavo *et al.* (1991) showed that embryogenic cell cultures consist of two types of cells: vegetatively proliferating cells that respond to increased levels of auxin in the medium by increasing their level of auxin-binding proteins (ABP) (they call this response ABP modulation) and proembryogenic masses (PEMs), which once generated, have lost their ABP-modulating capacity, and under appropriate conditions develop into embryos leading to regeneration of plantlets. Furthermore, Filippini *et al.* (1992) found that the capacity to modulate ABP levels from high to low levels occurred in a cyclical manner, and appeared to be a pre-requisite of embryogenic capacity. Cells having either a high or low level of ABP show severe reductions in regeneration efficiency due to their inability to change it in response to variations in exogenous auxin.

A large number of observations on various embryogenic tissue culture systems support a general concept, which emphasises the central role of hormone- and/or stress-induced activation of signal transduction systems. Consequently, the internally transmitted signals trigger substantial changes in chromatin structure, alteration of transcription and induction

of a series of cell divisions that lead to the formation of either dedifferentiated callus tissues or somatic embryos (Dudits *et al.*, 1995). Many of the genes involved in these events are auxin-inducible. In soybean hypocotyl tissues, Czarneka *et al.*(1984) discovered the activation of heat shock genes by 2,4-D treatment. Differential screening of a cDNA library of 2,4-D treated alfalfa (*Medicago sativa*) callus tissues resulted in the isolation of a 571 bp cDNA clone, *MsPRP5*, encoding a proline-rich protein (Gyöegyey *et al.*, 1997). The accumulation of *MsPRP5* mRNA is auxin concentration-dependent in differentiated callus tissue and this clone may represents a new type of proline-rich protein gene which responds to hormonal shock and possibly other stresses as well (Gyöegyey *et al.*, 1997). It is also not surprising that a very high number of clones (180 clones) were isolated by cDNA subtraction from alfalfa (Medicago falcata L.) leaves during the 2,4-D induction period (Russinova *et al.*, 1998).

Recent studies have revealed that in higher plants, heterotrimeric G proteins are involved in hormonal and light signal transductions in defence responses and in the regulation of ion channel activities (Ma, 1994; Bowler and Chua, 1994). Msgbl (Madicago sativa Gβ-like) has been cloned from alfalfa and shown to be expressed in all tissue types examined (McKhanm *et al.*, 1997). Most notably, the transcript level was highest in young embryos, composed of actively dividing cells, and decreased as embryos completed division and acquired their mature form. Besides this gene, *arcA* of tobacco has been well characterised to be auxin-inducible (Nagata *et al.*, 1994; Ishida *et al.*, 1996). The sequence determination of *arcA* revealed that it has homology to a β subunit of heterotrimeric G proteins. This gene product is possibly involved in maintaining the dedifferented state of plant cells by auxin. Using differential screening, three auxin-regulated genes, which were named *parA*, *parB* and *parC*, were isolated from tobacco mesophyll protoplasts cultures (Nagata *et al.*, 1994). The expression of these *par* genes could play a pivotal role in regaining the meristematic activity of the differentiated tobacco mesophyll cells.

1.11.2 Heat shock protein (HSP) genes

All organisms, including the higher plants, possess a set of stress-responsive genes that code for proteins with protective functions (eg heat shock). The synthesis of heat shock

proteins (HSPs) has been shown in tissues from various plant species (Schöffl *et al.*, 1988). Several members of various plant heat shock gene families have been cloned and structurally characterised (Neumann *et al.*, 1989). Sequence analysis revealed conservation of genes encoding the high-molecular-weight hsp70 polypeptides from plants and other eukaryotes (Wu *et al.*, 1988). Furthermore, multigene families are responsible for the synthesis of the relatively high abundance and distinct variants of low-molecular-weight HSPs (Mansfield and Key, 1987; Nagao *et al.*, 1985). An increasing amount of evidence suggests that in addition to the defence function during thermal shock, the HSPs have an important function in cell proliferation and differentiation (for review, Bienz and Schlesinger, 1987). For example, the expression of heat-shock genes occurred during embryogenesis in somatic cells of alfalfa and carrot (Dudits *et al.*, 1991; Kitamiya *et al.*, 2000). Pitto *et al.* (1988) observed characteristic differences in the patterns of heat shock proteins at different developmental stages of carrot embryogenesis. In carrot cultures, the globular stage somatic embryos accumulated considerably less heat shock protein mRNA in comparison with embryos at later stages, or to cultured callus cells after heat shock (Zimmerman *et al.*, 1989). A possible link between embryogenic response and heat shock proteins was revealed when one of the temperature sensitive non-embryogenic carrot mutants (ts59) turned out to be defective in the phosphorylation of a heat shock protein (Terzi and LoSchiavo, 1990b). Heat shock genes can be activated by 2,4-D treatment in soybean hypocotyl tissues (Czarnecka *et al.*, 1984). Analysis of the 3'-intergenic element of an auxin regulated gene cluster (SAUR genes) in soybean showed high homology to the sequence motif located 150 bp downstream of the stop codon in the soybean heat shock gene 6834 (McClure *et al.*, 1989). The functional significance of this homology remains unknown. In alfalfa (*Medicago sativa* L.), small heat shock genes (*Mshsp 18-1*; *Mshsp 18-2*) were found to be expressed in early, globular and heart stage embryos developed from somatic cells under normal culture condition (Györgyey *et al.*, 1991). The alfalfa HSPs share a homologous stretch of amino acids in the carboxy terminal region with hsp22, 23, 26 from *Drosophila*. This region contains the GVLTV motif which is characteristic of several members of small HSPs (Györgyey *et al.*, 1991).

Heat shock proteins may play a role during cell proliferation, differentiation and embryogenesis (reviewed by Bond and Schlesinger, 1987b). Under non-stress conditions,

heat shock proteins have important functions in cell proliferation (Pechan *et al.*, 1991). The cell-cycle regulation of HSPs has been documented by several studies (Sorger and Pelham, 1987; Celis *et al.*, 1988; Milarski *et al.*, 1989; Jentsh *et al.*, 1990). Inactivation of one of the heat-shock 70 genes (SSC1) in *Saccharomyces. cerevisiae* prevented cell division (Craig *et al.*, 1987). Heat shock has been reported to cause the activation of mitogen activated protein (MAP) kinase in animal systems. MAPK is activated by MAPK kinase via phosphorylation, enabling MAPK to translocate to the nucleus and phosphorylate transcription factors which allow cells to enter mitosis (Chen *et al.*, 1992; Dodeman *et al.*, 1997). Under stress conditions, HSPs have been suggested to interrupt the synthesis of other proteins while at the same time stabilizing proliferation-regulatory proteins by inhibiting the synthesis of enzymes required for their turnover, and moreover, interact with proteins involved in cell proliferation. Consequently, it is conceivable that cells not amenable to the cell-proliferation pathway could, under certain conditions, undergo this progress when HSPs appear in response to stress imposition (Pechan, 1991). HSPs can function as molecular chaperones (Ellis, 1990; Morimoto, 1998) to ensure proper folding and assembling of cellular proteins during a change in the developmental programme such as initiation of somatic embryogenesis (Dudits *et al.*, 1991, 1995).

1.11.3 Regulatory genes expressed during embryogenesis

A number of regulatory genes are involved in embryo development. The expression of a gene coding for the 'somatic embryogenesis receptor kinase' (SERK) was used as a marker to define embryogenic cells in the activated hypocotyls explants of carrot (Schmit *et al.*, 1997). In this case, a class of elongated cells on the explant surface was reported to be competent for embryogenesis. In another approach, in *Dactylis glomerata* leaf explants, using the expression of the *Dactylis SERK*-homologue gene as a marker of competence, exclusively small, isodiametric cells with rich cytoplasm proved to be competent and could develop into somatic embryos (Somleva *et al.*, 2000).

While ectopic expression of the *SERK* gene enhanced the embyogenic response of cultured cells induced to form somatic embryos, the ectopic overexpression of the *LEC1* gene of *Arabidopsis* induced embryo development directly on vegetative tissues in the

absence of any exogenous treatments (Lotan *et al.*, 1998). The *LEC 1* gene was identified by studies of a mutation causing defects in embryo maturation and desiccation (Meinke, 1992; Meinke *et al.*, 1994). Cloning and sequencing of this mutated gene revealed that it coded for a protein homologous to a subunit of a CCAAT box-binding transcription factor (Lotan *et al.*, 1998). Further studies indicated that among other roles, the protein might function during the early stages of embryogenesis in the suppression of suspensor proliferation (Lotan *et al.*, 1998). The *LEC 1* gene is expressed from the octant stage up to the late torpedo stage of embryogenesis. The expression of the *LEC 1* cDNA under the control of the 35S promoter in transgenic plants resulted in several abnormalities, which indicated that embryo-specific programs were not completely shut off or had been reinitiated in the seedlings (Lotan *et al.*, 1998).

More recently, another gene, the *LEC 2*, has been identified in *Arabidopsis*, having similar characteristics (Stone *et al.*, 2001). It also codes for a transcription factor, but has a plant specific B3 protein domain which is similar to the transcription factors viviparous1/ABA insensitive3 and fusca3, acting primarily in developing seeds. Ectopic, postembryogenic expression of *LEC* 2 in transgenic plants also resulted in the formation of somatic embryos and other organ-like structures to vegetative tissues (Stone *et al.*, 2001). Taken together, both the *LEC 1* and the *LEC 2* can be considered to be transcriptional regulators that can establish a cellular environment sufficient to initiate embryo development (Fehér *et al.*, 2003).

Another family of transcription factors, those containing the so-called MADS-box domain, are also important regulators of many plant developmental processes (Jack, 2001 a). One of the best-described examples of their importance in plant development is the regulation of flower development and floral organ identity (Jack, 2001 b). It was shown that the MADS-box containing AGAMOUS-like 15 (AGL-15) transcription factor accumulated in embryos of diverse origin, including zygotic, apomictic, microspore-derived and somatic embryos (Perry *et al.*, 1999). Using promoter-GUS gene fusions, it was found that its expression is not restricted to embryogenesis and it is more likely linked to a juvenile tissue state (Fernandez *et al.*, 2000). These results indicated that although this protein is associated with embryogenesis, it has a more general function in plant development. Recently, a cDNA of another MADS-box containing transcription factor, named the

BABYBOOM (*bbm*), was identified by investigating differentially expressed genes during *brassica* microspore embryogenesis (Boutilier *et al.*, 2000). When this cDNA was overexpressed under the control of the 35S promoter in transgenic plants, ectopic formation of embryos and cotyledons on leaves was observed. There are no data about its role during zygotic and somatic embryogenesis, but it can be supposed to have a general role during the different forms of plant embryogenesis (Fehér *et al.*, 2003). The detailed introduction of plant transcription factor will be described in chapter 4.

In this PhD project, a HD-Zip transcription factor gene isolated from alfalfa explant tissues was investigated. This gene is of particular interest because similar genes have been found to be associated with somatic embryogenesis in other systems. Further studies of this gene will provide important insights into the role of this HD-Zip gene in somatic embryogenesis.

1.11.4 Identification of embryogenic cells

It has been difficult to study the early stages of embryo development because the cells constituting the embryo are small, and few in number (Perry *et al.*, 1999). Good markers are therefore required for revealing specific cellular processes related to the somatic-to-embryogenic transition and the determination/description of cellular states (Fehér *et al.*, 2003). Several molecular markers have been used to distinguish between embryogenic and non-embryogenic cell cultures (Sterk *et al.*, 1991; De Jong *et al.*, 1992; Schmidt *et al.*, 1997; Wilde *et al.*, 1988; de Vries *et al.*, 1988; Dubois *et al.*, 1991; Heck *et al.*, 1995; Perry *et al.*, 1996; 1999). These include the *EP2*, DC3, *AGL15* and *SERK* genes; the monoclonal antibody JIM8 (Pennell *et al.*, 1992) and callose (Dubois *et al.*, 1991). In comparison with other markers, the SERK gene appears to be the most specific for embryogenic competence under culture conditions (Schmidt *et al.*, 1997). Until now, this is the only gene known to play a role in the acquisition of embryogenic competence in plant cells. *SERK* expression in carrot was shown to be characteristic of embryogenic cell cultures and somatic embryos, but its expression ceased after the globular stage (Schmidt *et al.*, 1997). It could also be detected in zygotic embryos up to the early globular stage, but not in unpollinated flowers or in any other tissue. Using the *SERK* promoter fused to

the luciferase gene and video cell tracking, it was shown that *SERK*-expressing single cells could develop into somatic embryos (Schmidt *et al.*, 1997). The *Arabidopsis* homologue of the carrot *SERK* cDNA has been cloned as one of five members of a small gene family (Hecht *et al.*, 2001). In Arabidopsis, *AtSERK1* expression was not restricted to embryogenic cells, but was characteristic of those cells capable of a rapid response to hormonal signals and competent to form somatic embryos or embryogenic cell cultures (Hecht *et al.*, 2001). Ectopic expression of the *AtSERK1* cDNA under the control of 35S promoter did not cause any specific phenotype. However, the efficiency of the initiation of somatic embryos was increased by approximately four-fold in the transgenic seedlings (Hecht *et al.*, 2001).

The expression of a SERK homologue was also tested in *Dactylis glomerata*, where somatic embryogenesis could be initiated directly from leaf explants, as well as indirectly from leaf-derived callus tissues (Somleva *et al.*, 2000). In this system, SERK occurred in small, cytoplasm-rich isodiametric cells that were shown to form somatic embryos by cell tracking. In contrast to carrot and *Arabidopsis*, *SERK* expression was maintained beyond the globular stages of embryogenesis in meristematic regions (Somleva *et al.*, 2000).

In the case of antibody JIM8, the presence of the JIM epitope was restricted to embryogenic cell cultures but cell tracking of cells labeled with this antibody failed to show a correlation with the ability of these cells to develop into somatic embryos (Toonen *et al.*, 1996). In contrast, the *SERK* gene showed a transient expression pattern (Schmidt *et al.*, 1997). The expression of the *SERK* gene was found to be very tightly correlated with the ability of cells of the correct morphology to attain the competent cell state. The application of AGL15-specific antibodies and immunohistochemistry have demonstrated that AGL15 accumulation and localisation are developmentally regulated during embryogenesis (Perry *et al.*, 1996; 1999). AGL15 was initially present in the cytoplasm of cells and became nuclear localised before or soon after embryogenic cell division began. A relatively high level of AGL15 is maintained in the embryo nuclei throughout the period of morphogenesis and then declines as the embryo matures (Perry *et al.*, 1996, 1999). AGL15 has been proposed to participate in the regulation of programmes active during the early stages of embryo development (Heck *et al.*, 1995; Perry *et al.*, 1996, 1999). Recently, much progress has been made in isolating genes encoding regulatory

factors expressed during early embryogenesis (Li and Thomas, 1998; Lotan *et al.*, 1998; Luerssen *et al.*, 1998; Mordhorst *et al.*, 1997; Berleth, 1998). However, relatively less is known about the corresponding gene products in terms of activity and/or regulation (Perry *et al.*, 1999). Further studies are required to reveal the link between cell competence for embryogenesis and specific gene expression.

1.12 Cell division and somatic embryogenesis

The control of cell division is an essential component of several stages of somatic embryogenesis. It appears to be necessary for dedifferentiation and redifferentiation. Acquisition of embryogenic competence largely relies on dedifferentiation, a process whereby existing transcriptional and translational profiles are erased or altered in order to allow cells to set a new developmental program. The activation of cell division is required to maintain the dedifferentiated cell fate, as well as for embryo differentiation. Cell division involves the control of plant body pattern formation and the further development, including somatic embryogenesis; The further description is given in section **2.1.6.**

1.13 The objectives of this PhD project

The main objectives of this PhD project were:

1. to investigate the relationship between the activation of cell division and the induction of somatic embryogenesis;
2. to determine the role of 2,4-D in the activation of cell division and the induction of somatic embryogenesis
3. to investigate the function of HD-Zip transcription factor Mfhb-1 in somatic embryogenesis in alfalfa.

CHAPTER 2 THE REGULATION OF CELL DIVISION DURING THE INDUCTION OF DIRECT SOMATIC EMBRYOGENESIS

2.1 Introduction

2.1.1 The plant cell cycle

Cell division is one of the most conspicuous features of living things. Its co-ordination with cell growth and differentiation is necessary to create complex multicellular organisms. This is achieved within the framework of a specific developmental plan that defines the characteristics of the particular organism (White-Cooper and Glover, 1995). Although elements of cell division regulation are conserved through the three domains that include prokaryotes and eukaryotes (Amon, 1998; Leatherwood, 1998; Mironov *et al.*, 1999), it is perhaps of most significance that cell division cycle in plants is most similar to that of other higher eukaryotes. Essentially, the DNA must be replicated and partitioned into two daughter cells following mitosis. The classical four-stage model of cell division cycle was first introduced in plants in 1953 (Howard and Pelc, 1953). The four stages of the cell cycle in the sequence G1-S-G2-M comprise: G1 (Gap 1 before DNA synthesis), S (DNA synthesis), G2 (Gap 2 after DNA synthesis) and M (cell division), and then on to the next cycle. For the convenience of description and experimentation, the M phase is further divided into five distinct stages: prophase, prometaphase, metaphase, anaphase and telophase, each characterised by a particular series of events. Eukaryotic cells may cease to divide if environmental and/or developmental signals require them to do so. Cells in the non-dividing state are termed quiescent (G0). Regulatory signals can divert cells in the G1 phase into a quiescent phase (G0) or stimulate quiescent cells to re-enter the cell cycle (Fowler *et al.*, 1998c).

2.1.2 The control of the cell cycle

The progress through the cell cycle is regulated at two principal control points (Van't Hof, 1985). One is situated at late G1 phase and the other at the G2/M boundary. A further

important control exists at the metaphase-anaphase transition and further subsidiary controls may also exist. Transit through these control points in eukaryotic cell requires activated kinase complexes. These normally consist of two proteins, a cyclin-dependent serine/threonine protein kinase (CDK) and a cyclin (Pines, 1995b). As mentioned below the actual proteins involved vary through the cell cycle. CDK activity is dependent on cyclin binding, which also determines substrate specificity and subcellular localisation of the CDK complex. The cell cycle is driven forward by the sequential activation and destruction of CDK activities, and this indicates that CDKs and cyclins play central roles in the regulation of cell cycle commitment and progression (Pines, 1995b).

2.1.2.1 Plant CDKs

Serine/threonine protein kinases are a super family of proteins that have a number of structural features in common. With the CDKs this is demonstrable in comparisons of the catalytic domains (Pines, 1994; Lees, 1995; Morgan, 1995; Nigg, 1995; Fowler *et al.*, 1998a). CDKs were first discovered during the genetic analyses of the cell cycle of budding yeast (Hartwell, *et al.*, 1974; Lörincz, *et al.*, 1984, Nasmyth, *et al.*, 1980) and fission yeast (Hindley, *et* al., 1984; Nurse, *et al.*, 1980) and in landmark studies, a CDK was found to be a component of *Xenopus* mitosis promoting factor (MPF; known as maturation-promoting factor at that time) (Dunphy, *et al.*, 1988; Lohka, *et al.*,1988).The first CDK to be described was encoded by the *cdc2* gene of *Schizosaccharomyces pombe* (Simanis and Nurse, 1986) and genetic analysis revealed the *cdc2* gene product, p34 ^{cdc2}. Subsequently, *cdc2* homologues have been isolated from many organisms. (reviewed by Jacobs, 1995). In yeast, a single CDK (*cdc2* in *Schizosaccharomyces pombe* or *CDC28* in *Saccharomyces cerevisiae*) governs both the G1/S and G2/M transitions (Nasmyth, 1996; Stern and Nurse, 1996). In animal cells, distinct CDKs that associate sequentially with different cyclins monitor cell cycle progression (Pines, 1996). Of the five mammalian CDKs strongly implicated in cell cycle control, three (CDC2/CDK1, CDK2, and CDK3) are closely related to the prototypical yeast *cdc2* and have the same characteristic PSTAIRE motif in the cyclin-binding domain (De Bondt, *et al.*, 1993). Two other CDKs, CDK4 and CDK6, form a distinct subfamily of CDKs in which PSTAIRE is substituted

with either PISTVRE or PLSTIRE, respectively. Both CDK4 and CDK6 are known to function exclusively in the G1 phase (Morgan, 1997). Like animals, plants have multiple CDK-related protein kinases, and five types of CDKs, namely CDKA through CDKE, have been defined according to phylogenetic, structural and functional similarities with animal and yeast CDKs (Joubès *et al.*, 2000a). This classification is mainly based on the conservation of the PSTAIRE motif in the cyclin-binding domain. The best characterised group CDKA comprises plant CDKs that are functional homologs of the yeast p34 $^{cdc2/cdc28}$ protein displaying the PSTAIRE canonical motif. Their expression and translation patterns are constitutive during the cell cycle (Colasanti *et al.*, 1991; Ferreira *et al.*, 1991; Fobert *et al.*, 1996; Hirt *et al.*, 1991, 1993). CDKB is found only in plants (Joubès *et al.*, 2000a) and possesses a divergent motif: either PPTALRE or PPTTLRE, reflecting the existence of two subgroups, CDKB1 and CDKB2, respectively. Both fail to functionally complement temperature-sensitive mutants of yeast CDC2/CDC28, suggesting some distinct functions in controlling the cell cycle (Lee *et al.*, 2003). CDKAs are supposed to regulate both the G1-S and G2-M transitions, whereas CDKBs regulate the G2-M transition (Mironov *et al.*, 1999) The three other CDK families (CDKC, D, and E) representing non- PSTAIRE kinases are poorly characterised and their function in the cell cycle regulation remains unclear.

2.1.2.2 Plant Cyclins

A large number of plant cyclins have been identified in various species (Renaudin *et al.*, 1996; Day and Reddy, 1998; de Veylder *et al.*, 1999; Sorrell *et al.*, 1999), of which about 30 genes for cyclin have been described in *Arabidopsis* (Vandepoele *et al.*, 2002). Based on their sequence similarities, plant cyclins have been classified into five major groups: A, B, C, D and H (Renaudin *et al.*, 1996; Yamaguchi *et al.*, 2000). The A- and B-type cyclins known as mitotic cyclins accumulate during the S, G2 and early M phase and during the G2 and early M phase, respectively (Mironov *et al.*, 1999). D-type cyclins control the progression through the G1 phase in response to growth factors and nutrients (Riou-Khamlichi *et al.*, 2000). C- and H-type cyclins have been characterised recently in poplar (*Populus tremula* X *tremuloides*) and rice (*Oryza sativa*; Yamaguchi *et al.*, 2000). Both

of them were found to interact specifically with the rice CDK-activating kinase, but only Orysa;CycH;1 could activate the kinase, suggesting that it is the effective regulatory subunit. A-, B-, and D-type cyclins were further categorised into smaller groups (Renaudin *et al.,* 1996), and Vandepoele *et al.* (2002) proposed 13 subgroups of *CycA1-3*, *CycB1-3* and *CycD1-7* in *Arabidopsis*. The phase-dependent expression of mitotic CycB is under transcriptional control (Mironov *et al.*, 1999). Recently, Ito *et al.,* (2001) have identified c-Myb-like proteins in tobacco plants, which control G2/M-phase-specific transcription of genes for CycB. However, functional analysis of mitotic cyclins is quite limited at the protein level, and their role in activation of cell division has not yet been clarified in plant tissues (Lee *et al.,* 2003)

2.1.2.3 The CDK catalytic subunit (CKS)

The typical CDK catalytic subunit (CKS) contains a 300 residues catalytic core that is completely inactive when it is monomeric and unphosphorylated (Morgan, 1995). The CDK catalytic core provides highly specific binding sites that allow the two substrates, ATP and protein, to nestle against one another in the desired orientation (De Bondt *et al.,* 1993; Morgan, 1996). Crystallographic studies on human CDK2 have shown a three-dimensional structure of the catalytic core which containing a smaller N-terminal lobe, dominated by a beta sheet and the large PSTAIRE helix, and a larger C-terminal lobe that is primarily helical. The unmodified CDK catalytic subunit is not capable of catalysing the phosphotransfer reaction and its activity is severely restrained by two mechanisms (De Bondt *et al.,* 1993). First, the substrate binding site is blocked by a large, somewhat flexible loop termed the T-loop (rises from the C-terminal lobe); and second, side chains in the ATP-binding site are oriented so that the ATP phosphates are poorly positioned for efficient phosphotransfer (Morgan, 1997).

2.1.2.4 The cyclin subunit

The cyclins are a remarkably diverse family of proteins, ranging in size from about 35 to 90 kDa. Distant members of the family often seem barely related at the primary sequence

level. Sequence homology tends to be concentrated in a 100-residue section known as the cyclin box, which is necessary for CDK binding and activation (Kobayashi *et al.*, 1992, Lees and Harlow 1993). Two groups have determined the crystal structure of a truncate cyclin A molecule (cyclin At) in which the N-terminal $\approx$170 residues have been removed to generate a minimal CDK-binding domain (Brown *et al.*, 1995, Jeffrey *et al.*, 1995). The crystal structure of human cyclin H, a small and distant member of the cyclin family, has provided clues about the features of cyclin structure that are likely to be conserved among all cyclins (Kim *et al.*, 1996; Andersen *et al.*, 1997). Crystallographic analysis has also revealed the presence of cyclin-like fold in the RNA polymerase II transcription factor, TFIIB (Bagby *et al.*, 1995; Nikolov *et al.*, 1995). Based on comparisons of primary sequences, Gibson *et al.*, (1994) proposed that cyclins may have evolved from an ancestral TFIIB-like molecule, raising fascinating questions about the evolutionary origins of cell cycle control mechanisms.

2.1.2.5 CDK activation by cyclin binding

Activation of a CDK requires association with a cyclin subunit. Homology among cyclins is often limited to a relatively conserved domain of about 100 amino acids, the cyclin box, which is responsible for CDK binding and activation (Kobayashi *et al.*, 1992; Lees and Harlow, 1993; Peeper *et al.*, 1993; Sherr, 1993; Lees, 1995), and, in part at least, defines the group of cyclins to which the sequence belongs (Pines, 1996). The biochemical features of the CDK-cyclin interaction vary in different complexes. Some CDK-cyclin pairs, such as human CDC2-cyclin B, CDK2-cyclin A, and CDK2-cyclin E, interact with high affinity in the absence of other components or modifications (Desai *et al.*, 1995). Some complexes, such as CDC2-cyclin A and CDK7-cyclin H, do not bind tightly unless the CDK subunit is phosphorylated at the activating threonine residue (Ducommun *et al.*, 1991, Desai *et al.*, 1995; Fisher *et al.*, 1995). The crystal structure of the human CDK2-cyclin A complex (Jeffrey *et al.*, 1995) has shed much light on the mechanism underlying cyclin binding and cyclin-dependent changes in CDK activity. As expected from the high affinity of this complex, the CDK2-cyclin A binding interface exhibits an unusually large surface area. Several helices in cyclin A contact both lobes of CDK2 in the region

adjacent to the active site cleft. Major contributions to binding are provided by interactions between the large PSTAIRE helix in CDK2 and helix 3 and 5 in the cyclin box domain of cyclin A; another major interaction involves the C-terminal lobe of CDK2 and the non-conserved N-terminal helix of cyclin A (Morgan, 1997). Enzymatic measurements of the monomeric CDKs versus the cyclin-bound forms have indicated that cyclin binding leads to a 40,000-fold increase in kinase activity (Connel-Crowley, 1993). The crystal structure studies on cyclin A-CDK2 have provided a structural basis for the activation process, which is likely to be applicable to all cyclin-CDK interactions. When the cyclin A-CDK2 complex structure is compared with that obtained previously for monomeric CDK2 (De Bondt *et al.*, 1993), it is evident that the binding of cyclin A causes dramatic structural changes within CDK2 (Jeffrey *et al.*, 1995). Conformational changes in the conserved cyclin-binding "PSTAIRE" region of CDK2 result in the rearrangement of the catalytic residues within the active site, specifically bringing Glu51 into alignment with Lys33 and Asp145. The triad configuration of these residues is essential for ATP phosphate orientation and magnesium co-ordination within the catalytic cleft. In other eukaryotic kinases, these three residues are naturally in the triad configuration and do not depend on the binding of a regulatory subunit for proper alignment. The change in the CDK2 PSTAIRE region is further coupled to a significant shift in its inhibitory 'T' loop domain (Jeffrey *et al.*, 1995). This shift removes the blockade imposed by the T loop structure on the catalytic cleft and further exposes the Thr 160/161 (Thr 161 in human CDC2, Thr 160 in CDK2) residue within the loop, thus making it more accessible for phosphorylation by the CDK-activating kinase, CAK (reviewed by Lees, 1995).

2.1.3 Other components involved in plant cell cycle control

Regulation of the cell cycle machinery at other levels than CDK or cyclin levels also exists in plants. It includes post-translational modification by phosphorylation, interaction with other cell cycle regulators (CDK inhibitors-CKI), proteolytic degradation, and via the retinoblastoma pathway and E2F-Dpa transcription factors, and differential subcelluar localisation. Research in these areas is still in progress in plants, although there has been a considerable amount of information accumulated in other systems.

2.1.3.1 Phosphorylation and dephosphorylation during the cell cycle control

Full activation of the mitotic CDK activity requires not only cyclin association but also specific phosphorylation- dephosphorylation events (Figure 2.1). So far most of the basic work in this area has been done in yeast / humans but there is now evidence emerging from plants. The additional regulatory mechanisms are dephosphorylation at the inhibitory phosphorylation sites (Thr 14 and Tyr 15 in human CDK2) by a dual-specificity phosphatase, CDC25, and phosphorylation of a critical threonine residue within the T-loop region (Thr 161 in human CDC2, Thr 160 in human CDK2), which is accomplished by another protein kinase called CAK (CDK-activiating kinase) (Coleman and Dunpohy 1994; Lees, 1995; Morgan, 1995; Fowler *et al.*, 1998a). CAK is a multimeric enzyme complex which is the trimeric CDK7- CycH- Mat1 complex in metazoans or the single-subunit Cak1 in budding yeast (Kaldis, 1999). CDK7 levels do not oscillate; consequently CAK activity is constant throughout the cell cycle and is unlikely to be rate limiting for cell cycle progression (Brown *et al.*, 1994; Tassan *et al.*, 1994). Phosphorylation most likely affects the cyclin binding site, as it enhances the binding of some CDK-cyclin pairs; conversely, cyclin binding may enhance phosphorylation (Morgan, 1995).

Whereas phosphorylation of Thr 160/161 activates the CDK kinase, phosphorylation at two other residues (Thr 14 and Tyr 15) inhibits the activity of the kinase. These inhibitory phosphates are added by yet another kinase (called Wee1 in yeast). As shown in step 1 of Figure 2,1, action of Wee1 results in an inactive Cdc2 enzyme. The inactivated enzyme, however, can be activated by removal of two inhibitory phosphate group, which is accomplished by a dual-specificity phosphatase encoded by the *cdc25* gene in yeast (step 2, Figure 2.1). Thus, the Cdc25 phosphatase and the Wee1 kinase act as competitors of one another, one stimulating Cdc2 and the other inhibiting its activity. Homologous versions of each of these enzymes occur in mammalian cells. Recent studies revealed that CAKs, CycH and Wee1 homologues have been isolated in plants (for review, Stals and Inzé, 2001). Evidence indicates that competition between Wee1 and Cdc25 activities become a critical determinant of the onset of mitosis. The activity of the products of both these genes is, in turn, regulated by other kinases and phosphatases, so that the system that

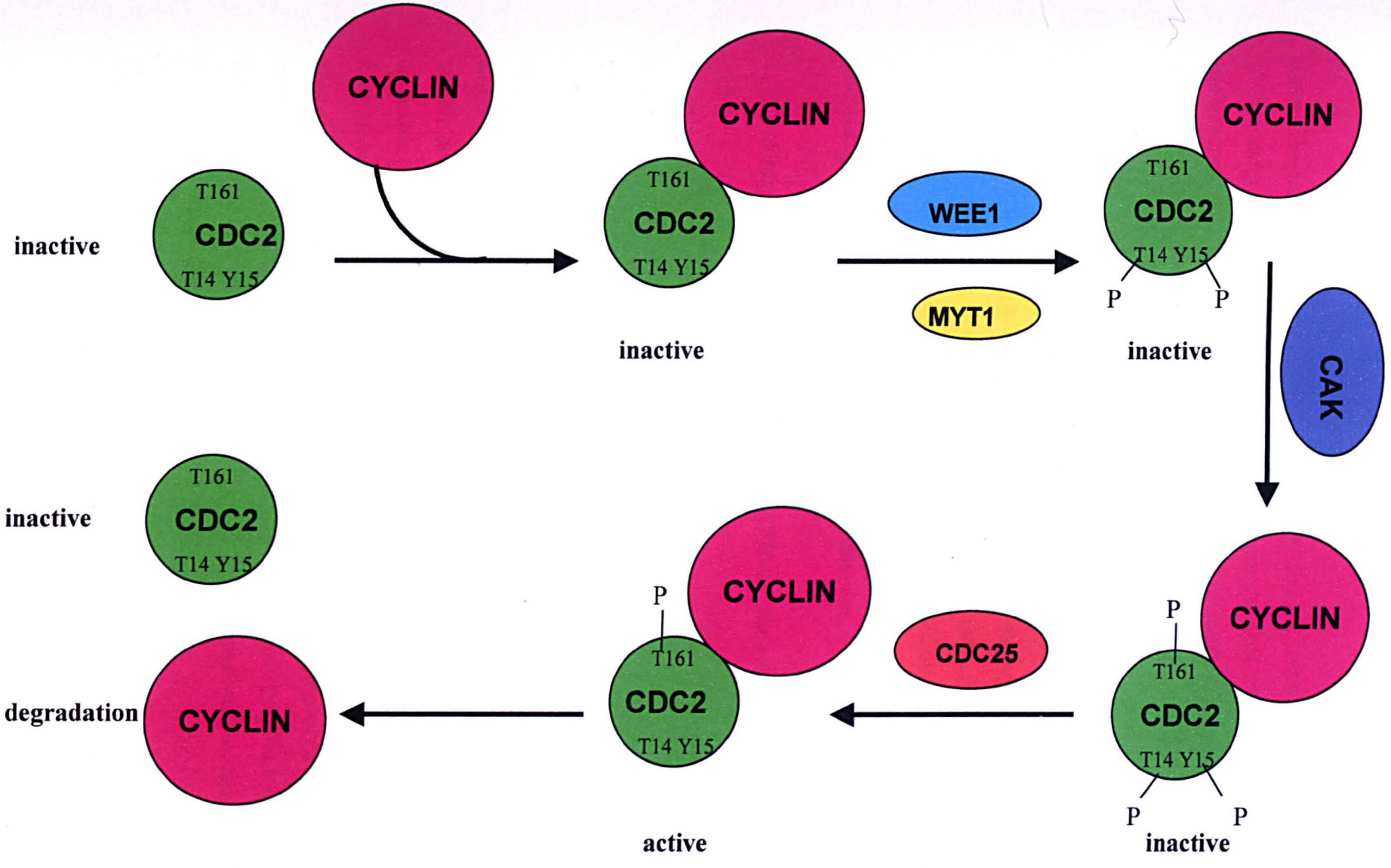


Figure 2.1 Model for regulation of Cdc2 kinase activity in fission yeast by cyclin, Cdc2-activating kinase (CAK), Wee1 and Cdc25. Progression through the cell cycle requires the phosphorylation and dephosphorylation of certain critical residues. Wee1 and CAK are proteins, and Cdc25 is a protein phosphatese. Cdc2 is active only when it is monophosphorylated at Thr 161.

control the cell cycle is quite complex (Morgan, 1995; Lees, 1995; Colemn and Dunphly, 1994; Fowler *et al.*, 1998a; Karp, 1999). Furthermore, the CDC25 can be phosphorylated and activated by CDC2 and CDK2, forming a positive-feedback loop to amplify CDK activity. Positive feedback may also be achieved by co-ordinated effects on other components: CDC2 may stimulate the kinase that inactivates Weel and inhibit the phoshatase(s) that inactivates CDC25 and activates Weel (Morgan, 1995).
So far, the CDC25 homologue of plants has not been cloned yet and the completion of the Arabidopsis genome-sequencing program showed that Arabidopsis does not have a CDC25 homologue (The Arabidopsis Genome Initiative, 2000). However, several lines of evidence from current studies highlighted that an unidentified dual-specificity phosphatase could be responsible for the dephosphorylation of the inhibitory Thr/Tyr residues in plants (Mészáros, *et al.*, 2000; Zhang *et al.*, 1996; McKibbin *et al.*, 1998).

2.1.3.2 Mitogen-activated protein kinases (MAPKs) in the cell cycle

Mitogen-activated protein kinases function in growth-factor signal transduction pathways upstream of the cell cycle machinery during G1, as well as downstream of it, in the M phase, to phosphorylate microtubule-associated proteins (MAPs). Several homologues of mitogen-activated protein kinases have been cloned from many species in plants, like alfalfa, tobacco, pea and *Arabidopsis* (Mizoguchi *et al.*, 1994; Bögre *et al.*, 1999). An alfalfa MAPK, MMK3 is activated late in plant mitosis and becomes localised to the plane of cell division (Bögre *et al.*, 1999). *Arabidopsis* mitogen-activated protein kinase is activated in extracts from auxin treated plants, suggesting that this class of kinase transduces mitogenic signals in plants (Mizoguchi *et al.*, 1994). Mitogen-activated protein kinase phosphorylation cascades may link oxidative stress responses to auxin signaling and cell cycle regulation (as reviewed by Hirt, 2000). For example, the tobacco MAPK Kinase Kinase (MAPKKK), NPK1, was shown to be involved in oxidative stress response, auxin signaling and cell cycle regulation (Hirt, 2000).

2.1.4 Inhibitors in cell cycle studies

Our knowledge of the molecular events that control the cell cycle has advanced considerably during the past few years through exploitation of highly synchronised plant cell suspensions, which provide a simple model to study proliferation in plants. Highly synchronised cells are powerful and effective tools for molecular analysis and for study of the biochemical events which occur during the cell cycle in plants. Normally, plant cell suspensions can be synchronised by chemical agents, which arrest the cell cycle by acting on the driving forces of the cell cycle engine, such as cyclin-dependent kinase activity, enzymes involved in DNA synthesis or proteolysis of cell cycle regulators, or by acting on the cell cycle apparatus (mitotic spindle).

2.1.4.1 Considerations for synchronisation

Several considerations have to be taken into account when attempting to synchronise cell systems. The drug action should be phase-specific and the chemical agent should be efficient at low concentrations and rapidly effective, to avoid abnormalities in subsequent phases. It is important to be sure that the cell division arrest is not a result of cellular death, the block must be reversed when the drug is removed. The optimal concentration of a chemical inhibitor, the duration of the treatment and time to re-enter the cell cycle have to be calibrated for each plant species. A plant cell suspension developed for synchronisation has to grow as fast and homogeneously as possible because the quality of the synchrony depends on the features of the cell suspension. The balance between toxicity and efficacy prevents the use of high concentrations of chemical inhibitors. Therefore, a one-step treatment can be improved by combining different inhibitors in a two-step blocking method. The synchronized cells obtained after the first drug treatment are further treated with a second drug to induce an arrest in the subsequent phase.

2.1.4.2 Inhibitors used in plant cell cycle studies

The specificity of action of the most frequently used chemical agents is given in Table 2.1. (Planchais *et al.*, 2000). The specific inhibitory actions of the chemical agents are illustrated during the cell cycle in Figure 2.2.

Table 2.1 Different type of cell cycle inhibitors and their mode of action (Modified from Planchais *et al.*, 2000)

Name	Target/mechanism	Block	Reversibility
Hydroxyurea	Ribonucleotide reductase	G1/S progression	+/−
Aphidicolin	DNA polymerase α and δ	G1/S progression	+
Colchicine	Microtubule depolymerisation	metaphase	+/−
Oryzalin	Microtubule depolymerisation	metaphase	+/−
Propyzamide	Microtubule depolymerisation	metaphase	+/−
APM	Microtubule depolymerisation	metaphase	+/−
Mimosine	Ribonucleotide reductase	G1, before initiation of replication	+/−
Olomoucine	CDK activity	G1/S and G2/M	+
Roscovitine	CDK activity	G1/S and G2/M	+
MG132	proteasome	Metaphase/anaphase	−

Note: +, reversible arrest; −, irreversible arrest; +/− reversibility dependent on the duration of the treatment.

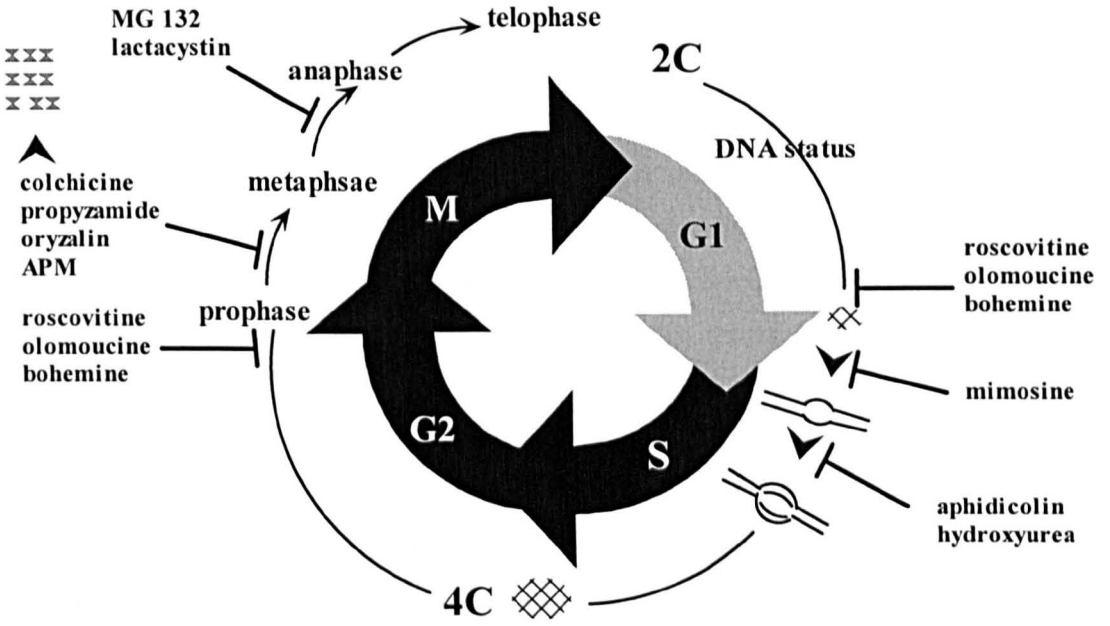


Figure 2.2 Chemical agents arrest the cell cycle progression at different points.

2.1.4.3 Inhibitors used in this study

Oryzalin is a dinitroaniline herbicide that has strong binding affinity for plant tubulins and inhibits microtubule polymerisation (Morejohn, *et al.*, 1987). This drug was found to arrest cells at metaphase (Verhoeven, *et al.*, 1990). At the onset of mitosis, the microtubule network is essential for spindle organisation and chromosome movement. Microtubules are dynamic polymers composed of tubulin. Inhibition of microtubule polymerisation is one of the key actions of anti-mitotic drugs, which act at the metaphase/anaphase transition (Planchais *et al.*, 2000). Large quantities of plant chromosomes can be obtained by the use of cell cultures that are synchronised at mitosis with chemical agents that disturb microtubule polymerisation (Conia, *et al.*, 1987).

DNA replication depends on the synthesis of deoxyribonucleotides and the activity of the enzymes of the replication machinery. Hydroxyurea (HU) inhibits the activity of ribonucleotide diphosphate reductase, thus depriving the cells of newly synthesised deoxyribonucleotide triphosphates, consequently preventing DNA replication (Young, *et al.*, 1964). In mammalian cells, HU does not result in synchronisation at the G1/S

boundary at low concentrations whereas, at high concentrations, HU is toxic to S phase cells (Pedrali-Noy, *et al.*, 1980). HU treatment is more efficient when applied to protoplasts than to entire plants. The quantity of inhibitor required to block activity in whole plant organs is often 10-fold higher than that required for plant cells in suspension culture. For example, 5 mM HU is needed to synchronise alfalfa cell suspensions in S phase (Magyar, *et al.*, 1993) whereas 100 mM HU is needed for *Arabidopsis* roots (De Almeida Engler, *et al.*, 1999; Ferreira, *et al.*, 1994). Moreover, the release from HU allows the synchronisation at subsequent phase. Eight hours after the release from a HU block, 55% of mitotic synchrony was observed in root tips of *V. faba* (Lucretti and Dolezel, 1995).

In my study, in order to determine the relation between the cell cycle reactivation and the embryogenic response in alfalfa, the cell cycle inhibitors oryzalin and HU were integrated into the induction stage of somatic embryogenesis.

2.1.5 Cell division and plant development

Cell division has long been considered to play a significant role in plant growth and development. Studies of various morphogenetic processes in plant development have often involved the determination of patterns and frequencies of cell division. The plant body is formed through the processes of growth, morphogenesis and cellular differentiation. Cell division is primarily responsible for generating plant growth and results in organs with particular shapes and sizes (together with cell expansion) and various cellular types (cell differentiation). First, due to the presence of a rigid cell wall, plant cells cannot move and subsequently organogensis is dependent on cell division and cell expansion at the site of formation of new organs (Lieven De Veylder *et al.*, 1996). Secondly, the organs of mature plants are not formed in miniature in the embryo but are derived from post-embryonic development and originate from the activities of small groups of cell, called meristems. The most important of these are the shoot apical meristem (SAM) and root apical meristem (RAM), located at the primary growing point of shoots and roots, respectively. Meristems remain active throughout the life cycle of plant and are responsible for the formation of plant parts. Thirdly, plants often respond to

their environment by changes in the pattern or rate of development (den Boer and Murray, 2000a).

Because plant development is tightly connected with cell division, links between the external environment and the control of cell cycle are to be anticipated. Hemerly *et al.* (1999) proposed that the control of cell division in plants is at two distinct levels. First, it involves the basic molecular controls of both the cell cycle and the developmental program, which governs particular rates and orientations of cell division. Secondly, these regulatory mechanisms must be interconnected and linked to an adaptable cell cycle.

Progression through the cell cycle in plants, as well as in other eukaryotes, is associated with the phase-specific transcription of defined sets of genes (McKinney and Heintz, 1991). This may contribute to an orderly progression through the cell cycle by ensuring that key proteins are produced in a strict temporal sequence. In most cases, cell-cycle-dependent changes in transcript levels are regulated by promoter activity (Koch and Nasmyth, 1994; Müller, 1995; Ito, 1998). The control of cell cycle progression is mainly exerted at two transition points: one late in G1, before DNA synthesis, and one at the G2/M boundary. G1/S-phase-specific transcription is regulated by mechanisms involving the E2F/DP heterodimeric transcription factor in animal cells (Black and Azizkhan-Clifford, 1999). The recent identification of the plant homologues of E2F (Ramirez-Parra *et al.*, 1999; Sekine *et al.*, 1999; Albani *et al.*, 2000; Vandepoele *et al.*, 2002) and their partner proteins DP (Magyar *et al.*, 2000; Ramirez-Parra and Gutierrez, 2000) supports the idea that plants have evolved a mechanism for G1/S-phase-specific transcription that relies on genes analogous to those acting in animal cells (Ito *et al.*, 2001; Vandepoele *et al.*, 2002).

Rb is a tumour-suppressor protein which may play an important role in controlling the onset of cell division. In its hypophosphorylated form, Rb is complexed with E2F-type transcription factors which are known to promote expression of S-phase-specific genes. Binding of Rb to E2F thereby prevents S-phase induction. Phosphorylated Rb is unable to form complexes with E2F transcription factors and allows DNA synthesis. All D-type cyclins show a specific amino acid motif (LXCXE) permitting them to bind Rb. After the initial isolation of the first plant D-type cyclins, it became clear that they share the common defining structural features of a cyclin box, a domain involved in CDK binding,

and an Rb binding motif. By analogy with animal D-type cyclins which interact with, and phosphorylate, Rb to control G1 progression, plant CycD may act in the G1/S transition according to a generally conserved mechanism in higher eukaryotes. This model would predict that CycD expression responds positively to mitogenic signals promoting cell division, and then targets its cognate kinase activity to the Rb protein. Upon the hyperphosphorylation of Rb, E2F transcription factor is activated which allows or induce expression of genes involved in processes leading to cell cycle progression and S-phase entry (Dewitte *et al.,* 2003 Florida 2002) (Figure 2.3). A considerable body of largely circumstantial evidence supports this model, including the finding that many genes likely to be required for S-phase entry possess E2F-binding sites that at least in a few cases have been shown to be involved in their cell cycle regulation. Other evidence supporting a role for CycD' in G1 progression and/or the G1/S transition comes largely from work on suspension cultures. In an *Arabidopsis* cell suspension culture showing partially synchronous re-entry into cell cycle after sucrose starvation, the level of CycD3- and CycD2-encoding mRNA increased from a low level during early (CycD2) or late G1 phase (CycD3) (Riou-Khamlichi *et al.,* 2000), suggesting that transcriptional control plays a part in the regulation of both genes' activity. Immunoprecipitation experiments with antisera against CycD2 and CycD3

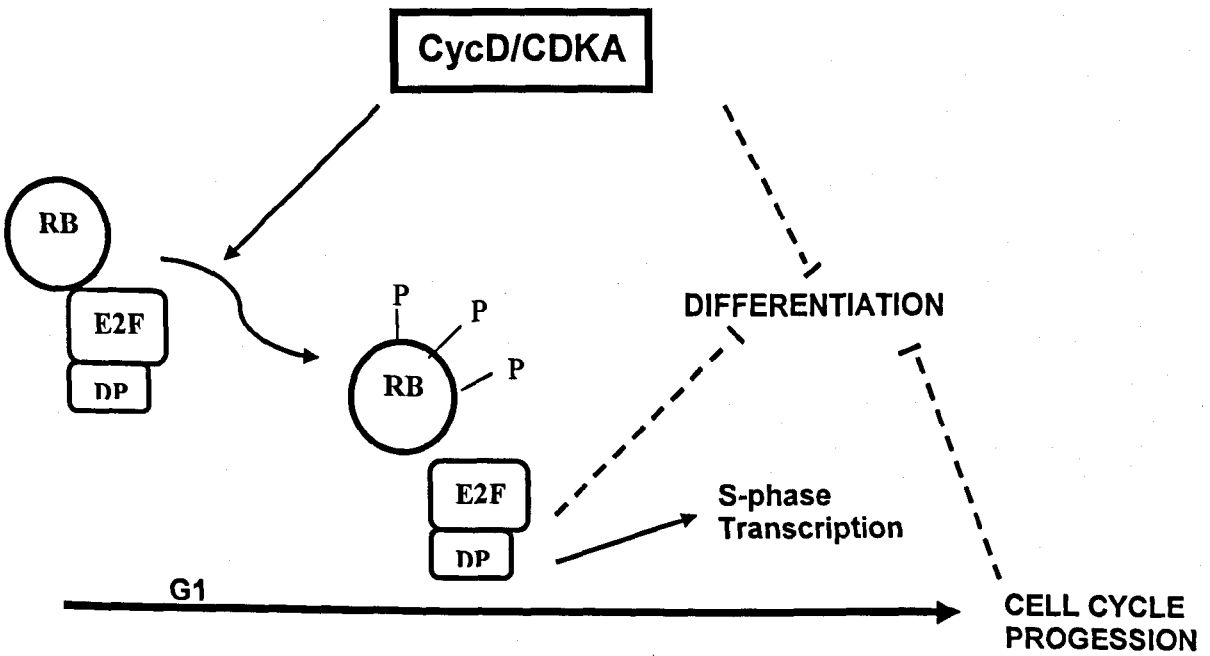


Figure 2.3 A proposed role for D-type cyclins and their cognate kinase complexes in integrating the interlinked processes of cell cycle progression and differentiation in response to morphogenetic and physiological signals. D-type cyclins form active complexes with CdkA, and phosphorylate Rb protein. Active E2F/DP heterodimeric factors can then activate cell cycle processes leading to S phase entry.

proteins revealed that these cyclins interacted *in vivo* with the archetypal PSTAIRE-containing the CdkA;1 (Healy *et al.*, 2001). Transcriptional regulation of both the *CYCD2;1* and *CYCD3;1* genes in *Arabidopsis* support the idea that for *CYCD2* and *CYCD3* there are at least strikingly different modes of regulating their activity, and possibly therefore differences in their biochemical roles (Dewitte *et al.*, 2003; Florida 2002). In *Arabidopsis*, three *E2F* genes have been identified (de Jager *et al.*, 2001), and further related genes also exist. E2F proteins form active heterodimeric complexes with DP proteins, and this interaction confers their nuclear translocation and transactivation (De Veylder *et al.*, 2002; Kosugi and Ohashi, 2002). *In situ* hybridisation analysis of D-type cyclins showed differential expression of the different D-type cyclins (De Veylder *et al.*, 1998; Gaudin *et al.*, 2000). Regulation of D-type cyclin gene transcription conferred by their promoters, suggesting that the expression of at least some *CYCD* genes is not simply proliferation-associated (and hence in all dividing cells), but is subject to tissue-specific or developmental control (Dewitte *et al.*, 2003; Florida 2002). Overexpression experiments of D-type cyclins induced significant effect on growth and development. Overexpression of *Arabidopsis* CDC2;1 shortened the G1 phase in tobacco meristems and hence promoted overall growth rate, but did not alter development (Cockcroft *et al.*, 2000). Constitutive overexpression of both CYCD3;1 and CYCD1;1 in *Arabidopsis*, increased cell numbers in leaves and perturbed cellular differentiation (Dewitte *et al.*, 2003; Florida 2002). The control of cell proliferation in a developmental context is likely to focus primarily on regulation of the G1 phase as main period of commitment. During this period, D-type cyclins interact with Rb proteins, E2F and their partner proteins DP to control the speed and behaviour of cells as they transit this critical phase (Figure 2.3). D-type cyclins are rate- limiting components of this progress. Substantial evidence now

supports the role of CYCD/CDK pathway in controlling entry into the cell cycle and indicates that the CycD/CDK pathway is a putative target for molecular mechanisms regulating both cell numbers and differentiation in organ formation. The much larger divergence found between D-type cyclins compared with A- and B-type cyclins might reflect the presumed role of D-type cyclins in integrating developmental signals and environmental cues into the cell cycle. For example, D3-type cyclins respond to plant hormones, such as cytokinins and brassinosteroids, whereas CYCD2 and CYCD4 are activated earlier in G1 and react to sugar availability (for review, see Stals and Inzé, 2001). Because of the large number of various D-type cyclins with different responses to developmental and environmental signals, cell division and growth in sessile plants might be more flexible than is observed in other eukaryotes (Vandepoele *et al.*, 2002). Later in the cell cycle, during G2- and M-phases, another set of genes is expressed. During G2-phase, B-type cyclins interact with and activate the cyclin-dependent kinase, which is critical for entry into mitosis (Pines and Hunter, 1990). Plant B-type cyclin genes, B1 and B2 classes, are expressed specifically in late G2- and M-phase (Mironov *et al.*, 1999; Ito, 2000). Unscheduled expression or overexpression of B-type cyclin genes often results in the formation of human cancers (Gong *et al.*, 1994), increased growth of plant organs (Doerner *et al.*, 1996) or lethal phenotypes in yeast (Lew *et al.*, 1991). In plants, periodic expression of B-type cyclin genes is regulated, at least in part, by a periodic change in the activity of their promoters in the cell cycle (Shaul *et al.*, 1996; Ito *et al.*, 1997; Colón-Carmona *et al.*, 1999; Tréhin *et al.*, 1999). Promoter analysis of the *Nicotiana sylvestris* cyclin B1 gene, *Nicsy;CYCB1;1*, showed the presence of a 23-bp element that acts as a cell cycle phase-independent transcriptional activator (Tréhin *et al.*, 1999). This element may be important for "quantitative" control, which determines the level of transcription, but is not associated with "qualitative" control, which determines the timing of transcription. Analysis of the promoter of the cyclin B1 gene, *Catro;CYCB1;1* (CYM), from *Catharanthus roseus* showed that the timing of promoter activation during the cell cycle is determined by a single type of *cis* element called MSA (M-specific activator), which is necessary and sufficient for periodic promoter activation (Ito *et al.*, 1998a, 2001). MSA-like motifs are found in B-type cyclin promoters from various plant species. The tobacco kinesin-like protein genes *NACK1* and *NACK2*, which are expressed with timing

similar to that of B-type cyclin genes in the cell cycle, also contain MSA-like motifs in their promoters. This finding suggests that a defined set of G2/M-phase-specific genes might be co-regulated by a common MSA-mediated mechanism in plants (Ito *et al.*, 1998a).

The model proposed by Francis (1998) recognises the importance of a requirement for a minimum cell size which commits the cell to the next division, and emphasises that a second network of genes, marshalled by "cdc2", ensures that the cell is of the correct size for cell division. Hence, cell size is the morphological marker of an optimum level of those proteins that will activate and ensure completion of a normal mitosis. Through the Cdc2 kinase (MPF), there is at least one feedback control that checks on cell growth and ensures that DNA replication is complete. The deployment of an endogenous timer will dictate the acquisition of particular sizes that the cell should grow for the next checkpoint of the cell cycle. The model dictates that in plant meristems, tissue-specific sizer controls are operating at both late G1 and late G2 checkpoints. The end-result will be cells of different size and, hence, different titratable amounts of gene products which programme the cell for a particular pathway of differentiation. Hence a cell size requirement is a component of a developmental programme. As a consequence of changes in the regulation of the cell cycle, cell size alters to meet the demands of different tissue domains. It is clear that premature cell division resulting in reduced cell size can disrupt normal development. The need to understand the biochemical regulation of cell size and cell division has to be a major target to be met to test further the intricate relationships between cell-size-mediated processes in relation to the development of the higher plant.

2.1.6 Reactivation of cell division and embryogenic competence in plants

In attempts to understand the molecular levels of the transition from the somatic to embryogenic cell type, the regulators of the cell cycle have been commonly considered as key determinants during both dedifferentiation and embryo formation. Recent development in the field of plant cell cycle research provide some clues to their roles.(for review, Fehér *et al.*, 2003). The core of the cell cycle machinery is well conserved among eukaryotes, in spite of obvious differences that also exist (for review, John, 1996). It is

well accepted that the regulation of plant cell division is highly dependent on external signals affecting plant development and morphogenesis (Dudits *et al.*, 1998; Den Boer and Murray, 2000).

In plants, both the G1-S and G2-M phase transitions can be controlled by changes in environmental factors (Yang *et al.*, 1994; Beeckman *et al.*, 2001; Touraev *et al.*, 1996). Embryogenic competence of *in vitro* cultured somatic cells can be stimulated by various stress factors, such as osmotic pressure, chlorides of heavy metals, pH, low or high temperature, starvation, mechanical wounding of explants or high auxin level (Kiyosue *et al.*, 1993). One of the mechanisms involved in stress-induced embryogenesis highlights the importance of the interaction between auxin and stress signaling which results in acquiring embryogenic competence by broad cellular reprogramming manifested at different levels. (for review, Fehér *et al.*, 2003). Whatever the detailed mechanism is, stress treatment triggers expression of factor(s) which affect gene expression and cell cycle regulation and thus induce somatic embryogenesis. Identified transcription factors controlling stress-specific response genes (Aarts and Fiers, 2003) can help to elucidate and understand the stress response in plants and its relation to embryogenesis.

The progression of cells through the different phases of cell cycle is regulated primarily by the activity of different cyclin-dependent kinase (CDK) complexes in all eukaryotes, including plants (for reviews, Mironov *et al.*, 1999; Mészáros *et al.*, 2000). The expression of the gene coding for the kinase component of the complex (Cdc2-related kinase) is induced by auxin (Hirt *et al.*, 1991) and present not only in dividing plant cells, but also in division-competent cells (Hemerly *et al.*, 1993), thus representing a marker for the degree of differentiation. It is generally believed that plant cells *in vitro*, with few exceptions, require exogenous growth regulators, auxin and cytokinin, for sustained cell division. In embryogenic alfalfa leaf protoplasts, the Cdc2MsA protein re-appeared in response to auxin treatment; whereas the histone H1 phosphorylating activity of this protein was dependent on post-translational modifications that required the presence of cytokinin (Pasternak *et al.*, 2000). These modification may involve the de-phosphorylation of the CDK protein on Thr14/Tyr15 regulatory residues (this mainly operates at G2-M transition), or by the induction of the expression of the regulatory cyclin subunit cyclin D3 (at G1-S transition) (for review, John, 1996).

Acquisition of embryogenic competence largely relies on dedifferentiation, a process whereby existing transcriptional and translational profiles are erased or altered in order to allow cells to set a new developmental program. The activation of cell division is required to maintain the dedifferentiated cell state. In cell cultures, the dividing cells can follow alternative developmental pathways such as unorganised callus growth, root and shoot initiation or somatic embryo formation. In animal cells, cell cycle progression and differentiation are related but divergent processes and their regulation is linked by components having important regulatory roles near the G1/S cell cycle translation point (Gao and Zelenka, 1997; Studzinski and Harrison, 1999). Some of these components, such as the retinoblastoma (Rb) repressor proteins, the E2F-type transcription factors, as well as D-type cyclins and CDK inhibitors (CKIs), exist in plants as well (Gutierrez, 1998; Mironov *et al.,* 1999; Mészáros *et al.,* 2000; Stals and Inzé, 2001), and exhibit similar mechanisms. This is further supported by some experimental observations. In *Arabidopsis*, leaf aging and differentiation are associated with increased expression of the ICK1, CDK inhibitor protein (Wang *et al.,* 1998). In maize leaf, the regions of older differentiated cells contain more Rb protein than younger tissues (Huntley *et al.,* 1998). Ectopic expression of the cyclinD3 gene caused abnormal meristem and leaf development in transgenic plants (Riou-Khamlichi *et al.,* 1999). Overexpression of the same protein in calli induced differentiation and greening and prevented shoot regeneration (Riou-Khamlichi *et al.,* 1999). Although further evidence is required, it is not difficult to hypothesise that several key regulators of cell cycle/differentitation control play a key role in somatic embryogenesis through coordinated interactions with hormonal, environmental and developmental signaling pathways (for review, Fehér *et al.,* 2003)

2.1.7 The development and use of cell cycle gene promoter::gusA reporter constructs as molecular markers of cell division activity

In order to investigate the role of cell division in the induction of somatic embryogenesis, transgenic lines of alfalfa carrying molecular markers of cell cycle activity were used. The development and use of cell cycle reporter constructs required the transformation and regeneration of alfalfa plants with cell cycle gene promoter::*gusA* constructs was therefore

an important prerequisite for these studies. It is therefore appropriate at this stage to consider the technology of plant transformation.

2.1.8 The significance of plant transformation

Plant transformation has become widely adopted as a method to both understand how plants function and to improve crop plant characteristics. The capacity to introduce and express (or inactivate) specific genes in plants provides a powerful experimental tool, allowing direct testing of hypotheses that have been exceedingly difficult to resolve using other biochemical approaches (Coruzzi and Puigdomenech, 1994). A variety of transformation methods have been developed up to date and these have allowed many of the world's most important crop plants such as cereals, legumes and other recalcitrant dicots to be transformed.

2.1.9 Gene transfer methods

Transformation methods include indirect gene transfer *via* *Agrobacterium*-mediated transformation, and direct gene transfer by particle bombardment (biolistics) (Klein *et al.*, 1987; Christou *et al.*, 1988), electroporation (Shillito *et al.*, 1985; DeKeyser *et al.*, 1990), chemical treatment with PEG (poly-ethylene glycol) (Potrykus *et al.*, 1985; Negrutiu *et al.*, 1987), microinjection (Crossway *et al.*, 1986; De Laat and Blaas, 1987), UV laser microbeam (Weber *et al.*, 1988), silicon carbide fibres (Kaeppler *et al.*, 1990) and electrophoresis (Griesbach and Hammond, 1993). *Agrobacterium*-mediated transformation is based on utilizing *Agrobacterium,* a pathogen of dicotyledonous (broad-leafed) plants that transfer genes into the plant genome (Fraley *et al.*, 1983). Problems with using *Agrobacterium* to transform monocotyledonous plants (grasses) spurred on the development of other methods, the so-called 'direct gene transfer' methods. The major direct gene transfer method, particle bombardment (or biolistics), is a popular method used for the transformation of monocotyledonous plants in many laboratories, despite *Agrobacterium*-based protocols having subsequently been developed for the transformation of monocotyledonous plants (Slater, *et al.*, 2003). Among these methods,

Agrobacterium-mediated transfer, the first widely adopted means of creating transgenic plants, remains the most popular transformation technique. Compared with direct gene transfer, the greatest advantage of *Agrobacterium*-mediated transformation is that it permits the delivery of transgene into recipient cells at relatively high frequency without severe damage to the plant cells. Moreover once DNA is integrated into the plant genome, it does not normally undergo any major rearrangements and it integrates into the genome at a low copy number.

2.1.10 *Agrobacterium*-mediated transformation

Agrobacterium tumefaciens is a Gram-negative soil bacterium that can induce tumours called crown galls on many plant species. This is due to the natural capacity of this bacterium to introduce a segment of oncogenic DNA (T-DNA-transfer DNA), derived from its large tumor-inducing (Ti) plasmid into plant cells at wound sites. The transferred DNA is stably integrated into the plant genome *via* illegitimate recombination. The plasmid has a size of approximately 200 kb and for this reason is not very amenable to cloning strategies. To overcome this disadvantage, a binary vector system has been established by directed genetic engineering (Hoekeman *et al.*, 1983).
In general, with susceptible plants, *Agrobacterium*-mediated transformation is the easier and cheaper and thus most popular choice for producing transgenic plants. It also avoids problems of multiple gene copies which tend to be a feature of direct approaches. It offers the potential to generate transgenic cells at a high frequency, usually without significant problems in plant regeneration (Hansen, 1997).

2.1.11 Tumour-inducing (Ti) plasmids

Ti plasmids from different strains of *A. tumefaciens* generally share several common features, in that they contain: 1) one (or more) T-DNA regions; 2) a *vir* region; 3) an origin of replication; 4) a region enabling conjugative transfer and 5) genes for the catabolism of opines (octopine or nopaline). Nopaline strains of Ti plasmid have one T-DNA of approximately 20 kb, whereas octopine strains have two T-DNA regions, termed

"T_L" and "T_R", that are approximately 14 and 7 kb, respectively. However, only the T_L region is oncogenic (the T_R region carries genes for opine biosynthesis). The T-DNA region of any Ti plasmid is flanked by the presence of the right- and left-border sequences. These border sequences are 24-bp imperfect repeats. This region contains the genes that code for proteins involved in hormone biosynthesis (oncogenes), opine synthesis and for determining tumor size (Slater *et al.*, 2003).

Genetic analysis of the Ti plasmid reveals that two regions of this plasmid are essential for tumorigenesis; the T-DNA and the *vir* or virulence region, which is in an ~40-kb region outside the T-DNA and responsible for the transfer of the T-DNA region into the host plant. The 24-bp repeats border sequences play an important role in the transfer and integration of T-DNA into the plant genome. The transferred segment normally ends within or very close to these 24-bp sequences and any DNA sequences placed between these two borders will be efficiently transferred and stably integrated into the genome of the host plant. The 24-bp border repeats, in the correct orientation, are sufficient to promote DNA transfer when complemented by a functional *vir*-region. This property forms the basis for producing vectors for plant cell transformation (Herrera-Estrella and Simpson, 1988).

2.1.12 Binary vector systems

The binary vector system is based on the fact that the *vir*-region and the T-DNA need not be linked on the same replicon for functional T-DNA transfer to occur. In the binary vector system, two autonomously replicating plasmids are involved: a small binary (shuttle) vector that contains the T-DNA borders, and a disarmed helper Ti plasmid from which the oncogenic sequences have been removed but which supply the *vir* functions. The binary vectors have origins of replication for *Escherichia coli*, which is used as a host for molecular cloning, and for *A. tumefaciens*, into which the vectors are transferred by electroporation or triparental mating in order to be used in the infection and transformation of plants. The gene of interest and the selectable marker are inserted into the T-DNA flanked by short, conserved right and left border sequences. Foreign DNA sequences can be cloned into the T-DNA region relatively easily when it is on a small

plasmid. The transfer of the T-DNA to the plant is activated by the gene products of the *vir* region of the disarmed Ti plasmid acting in *trans*, which are activated by phenolic compounds exuded from wounded plant cells. The transferred DNA normally does not undergo major rearrangement processes, and in most cases is integrated as a single copy (Hansen, 1997). The standard protocols for transformation are described by Van Wordragen and Dons (1992) and Walkerpeach and Velten (1994). pBIN19 was one of the first binary plant transformation vectors (Bevan, 1984) and has been widely used since then.

2.1.13 Selectable markers

Plant transformation, using either *Agrobacterium* or particle bombardment, is a very-low frequency event. Only a small proportion of target cells typically receive the introduced DNA during these treatments, and only small a proportion of these cells survive the treatment and stably integrate introduced DNA (Franks and Birch, 1991; Grant *et al.*, 1991). It is therefore generally vital to detect or select for transformed cells efficiently among a large excess of untransformed cells (Birch and Bower, 1994), and to establish regeneration conditions allowing recovery of intact plants derived from single transformed cells (Walden and Wingender, 1995). In most cases this selection is based on the inclusion into the culture medium of a substance that is toxic to plants. Normally, some means for selecting the transformed plant cells is provided by the plant transformation vector. The selectable marker on the vector confers resistance to the toxic substance when expressed in transformed plant tissue. In many cases antibiotics can be used, as they efficiently inhibiting protein synthesis in the organelles, particularly the chloroplasts (Slater *et al.*, 2003). Therefore, antibiotic resistance genes can be used as selectable markers in plants. The most commonly used selectable marker gene is neomycin phosphotransferase (*nptII*), conferring resistance to the antibiotic kanamycin, to which most plants are sensitive. Other antibiotic resistance genes such as hygromycin phosphotransferase (*hpt*), dihydrofolate reductase (*dhfr*), phosphinothricin acetyl transferase (*bar*) and bleomycin resistance (*ble*), have also been widely used in plant transformation vectors. The development of alternative selectable marker genes also allows for the re-transformation of plant tissue

that already expresses one or more different selectable markers. Other potential selectable markers are also being investigated, these include: the reporter gene *GFP*, which can be used for the visual screening of transformants; the *Agrobacterium* isopentenyl transferase (*IPT*) gene, which determines growth and morphology during regeneration; and the use of cytokinin glucuronidase in combination with β-glucuronidase.

Initially there was only one selectable marker (*nptII*) for selecting transformed plants in pBIN19, located close to the right border (RB). The multiple cloning site (MCS) includes 8 restriction enzyme sites which are situated between the *EcoR*I and *Hind*III sites. The MCS is in the *LacZα* fragment, making blue/white screening possible. With the rapidly increasing application of transgenic technology to a wide range of plant species, the requirement for a more flexible set of binary vectors becomes obvious. Thus modern binary vectors tend to come in "families". These offer a choice of selectable marker (both antibiotic and herbicide resistance in many cases) and, in some cases, the opportunity for dual selection by incorporating two selectable markers on the one vector. This dual selection allows a much more efficient selection approach to be applied to plants, in which it has traditionally proved difficult to avoid false-positives during the selection process (Slater *et al.*, 2003).

2.1.14 The pBECKS400 series

The pBECKS400 vectors used in this study belong to a series of binary T-DNA vectors (pBECKS), which were developed by Dr A McCormac in the NBI laboratories (McCormac *et al.*, 1997). The pBECKS400 series was designed with a view to facilitating the insertion of gene sequences into the vector and corrected the undesirable features of the pBIN19 vector by incorporating an origin of replication for high copy number in *Escherichia coli* and a CaMV 35S promoter cassette with a polylinker containing several restriction sites. The inserted sequences are linked to a chimeric neomycin phosphotransferase (*npt*II) gene for the selection of plant transformants using amino glycoside antibiotics such as kanamycin or G418 (McCormac *et al.*, 1997). This *npt*II gene does not contain the deleterious mutation present in pBIN19 constructs (Yanofsky *et al.*, 1990; Frisch *et al.*, 1995) and thus encodes the fully functional product. Furthermore,

it is positioned next to the left border of the T-DNA in order to reduce the possibility of producing truncated sequences of novel genes within transformants. This design is based on the principle of polarity in T-DNA transfer. Transfer originates from the right border to the left border and ensures that the selectable marker is transferred after the gene of interest if the selectable marker is next to the left border. The extraneous DNA segment, such as the M13 *ori*, found in the T-DNA of pBIN19 (Frisch *et al.*, 1995; Fray *et al.*, 1994) is also omitted, so that the size of the DNA region destined for transfer to the plant is minimised. Besides the *ntp*II gene, there is another antibiotic-resistance gene (*spc/str*) on the plasmid backbone in order to permit alternative selection strategies within *Agrobacterium* hosts. In this chapter, pBECKS400.P-GUS construct containing *Bvcrk1-17* (and *Bvcrk1-18, 26* and *29*) was used for transformation of alfalfa. The success in obtaining transgenic alfalfa plants discussed in this chapter contributed to the further development of a useful system for alfalfa transformation. In chapter 4, therefore, another member of this serial binary T-DNA vectors family, pBECK400/6 was chosen as a donor vector for transformation with HD-Zip gene fragments constructs. The basic T-DNA cassette comprises direct repeats of the right and left borders from nopaline T-DNA and a chimeric *npt*II gene, which is fused, at the 5' end, with the −157 to +1 fragment of the *nopaline synthase* (*nos*) promoter and with the *nos* polyadenylation sequences at the 3' end. A 5' Tobacco Mosaic Virus Ω leader, which was isolated from the U1 TMV strain, is also included between the *nos* promoter and *npt*II to act as a translation enhancer (Gallie *et al.*, 1987). The plasmid pBECKS400 carries the RK2 *oriV*, and *oriT*, the origin of replication from pBR322 for high plasmid copy number in *E. coli* (Figure 2.4).

2.1.15 Reporter genes

Reporter genes are used in plant transformation for the analysis of promoter activities, for monitoring the efficiency of the selection system used, and for following the inheritance of foreign genes in subsequent plant generations. The ideal reporter genes should have low or no background activity in plants, should not interfere with the plant cell metabolism, and should be detectable using an inexpensive detection system that is sensitive, quantitative and simple to use. At present, only a small number of reporter genes are in

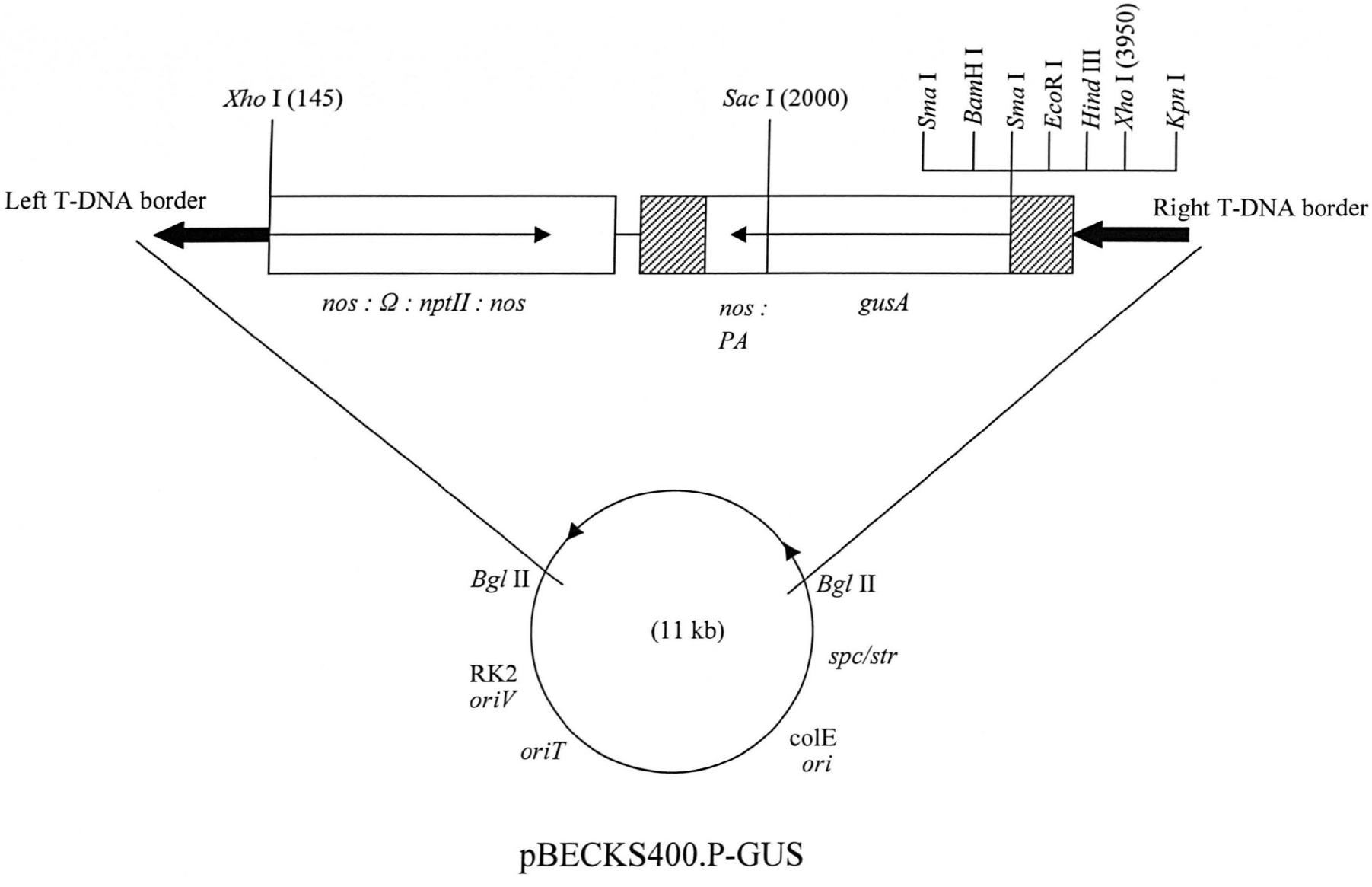


Figure 2.4 Schematic map of pBECKS400.P-GUS

widespread use in plant transformation vectors. The bacterial *uidA* (*gusA*) gene encoding the β-glucuronidase enzyme has been the most popular reporter gene in plants during last two decades. Other reporter genes, such as green fluorescent protein (*GFP*), luciferase genes (*lux* and *luc*) and, to a lesser degree (although it is widely used in animal systems), the chloramphenicol acetyltransferase gene (*cat*), have frequently been used (Slater *et al.*, 2003)

2.1.16 Arabidopsis cdc2a and CycB1;1 promoter::gusA reporter gene constructs

Arabidopsis CDC2a (P) and *CycB1;*1 (P) promoter::*gusA* reporter gene constructs have been used as molecular markers to investigate the regulation of cell division in a number of plant species (Hemerly, *et al.*, 1993;Ferreira *et al.*, 1994). The cyclin B reporter construct acts as a marker for actively dividing cells (specifically those progressing through G2 to M phase), whilst the cdc2a reporter construct is also expressed in non-dividing cells which have been described as "competent to divide" (Hemerly, *et al.*, 1993;Ferreira *et al.*, 1994). Transgenic alfalfa plants (*Medicago Falcata* L.) containing the *gusA* reporter gene controlled by the *Arabidopsis cdc2a* and *CycB1;*1 promoters were produced in the NBI laboratories (Shao *et al.*, 2000) and were used to investigate the induction of cell division competence and entry into the cell cycle during the early stages of somatic embryogenesis. High expression of *cdc2* (P) was found in both dividing cells, and also non-dividing cells that are competent to divide. In contrast, *CycB1;*1 (P) expression can only be detected in dividing cells. This experimental evidence indicated that the *Arabidopsis cdc2a* is good marker for cell division competence and *CycB1;*1 serves as suitable marker for cell division.

In addition to the *Arabidopsis cdc2a* (P) and *CycB1;*1 (P) constructs, the promoter constructs of a novel CDK-like kinase gene from sugar beet (*Bvcrk1*) were also used as markers of cell cycle activity.

2.1.17 The *Bvcrk1 gene*

Bvcrk1 (Beta vulgaris cdc2 related kinase) gene is a novel *cdc2*-like protein kinase genes. It has a consensus kinase core similar to all *cdc2*-like genes, but in particular to the *CHED gene* (Lapidot-Lifson *et al.,* 1992). Several genomic clones containing the complete coding region and the promoter region of the gene have been isolated. A cRACE (Maruyama *et al.,* 1995) approach has been used to identify the start site of transcription. Several of the products have been sequenced and one 5' terminal sequence has been regularly found. Analysis of the cDNA and its derived amino acid sequence indicates that *Bvcrk1* encodes a member of the CDKX subfamily of serine/threonine protein kinases. Comparison with other CDKs and CDKXs showed that BVCRK1 exhibited several features that allowed it to be classified as a novel CDKX. The PSTAIRE epitope in BVCRK1, ESVKFMARE, is highly divergent, the lack of any leucine/isoleucine residue being particularly interesting. This residue is very highly conserved in most CDKs/CDKXs as it is involved in cyclin binding. Subsequent genomic cloning and sequencing indicated that BVCRK1 is also much larger than the majority of CDKs due to the presence of large N- and C-terminal domains extra to the catalytic core. The role that these domains play is not known, though by analogy with mammalian sequences with similar domains, they may be involved in regulating activity, substrate binding or location. The C-terminus of BVCRK1, for example, contains several potential nuclear localisation sites (Milan, 2002).

Previous investigations of the expression of cell division-related genes in quiescent (G0) sugar beet cells showed that *Bvcrk1* expression was detected very early after the induction of cell division (Fowler *et al.,* 1998). *Bvcrk1* is therefore a potential marker for the early stages of the transition from G0 to the cell cycle. The *Bvcrk1* promoter has also been transformed into tobacco and carrot (Milan, 2000). The expression of the *Bvcrk1* promoter fragments showed patterns of activity predominantly in tissues undergoing active cell division, including apical and lateral meristems and vascular tissue. In the carrot storage root, the *Bvcrk1* promoter was expressed in the vascular cambial ring and adjacent vascular tissue (Milan, 2000). In this project, *Bvcrk1* promoter constructs were introduced into alfalfa plant to facilitate the investigation of cell division activation during the induction of somatic embryogenesis in alfalfa.

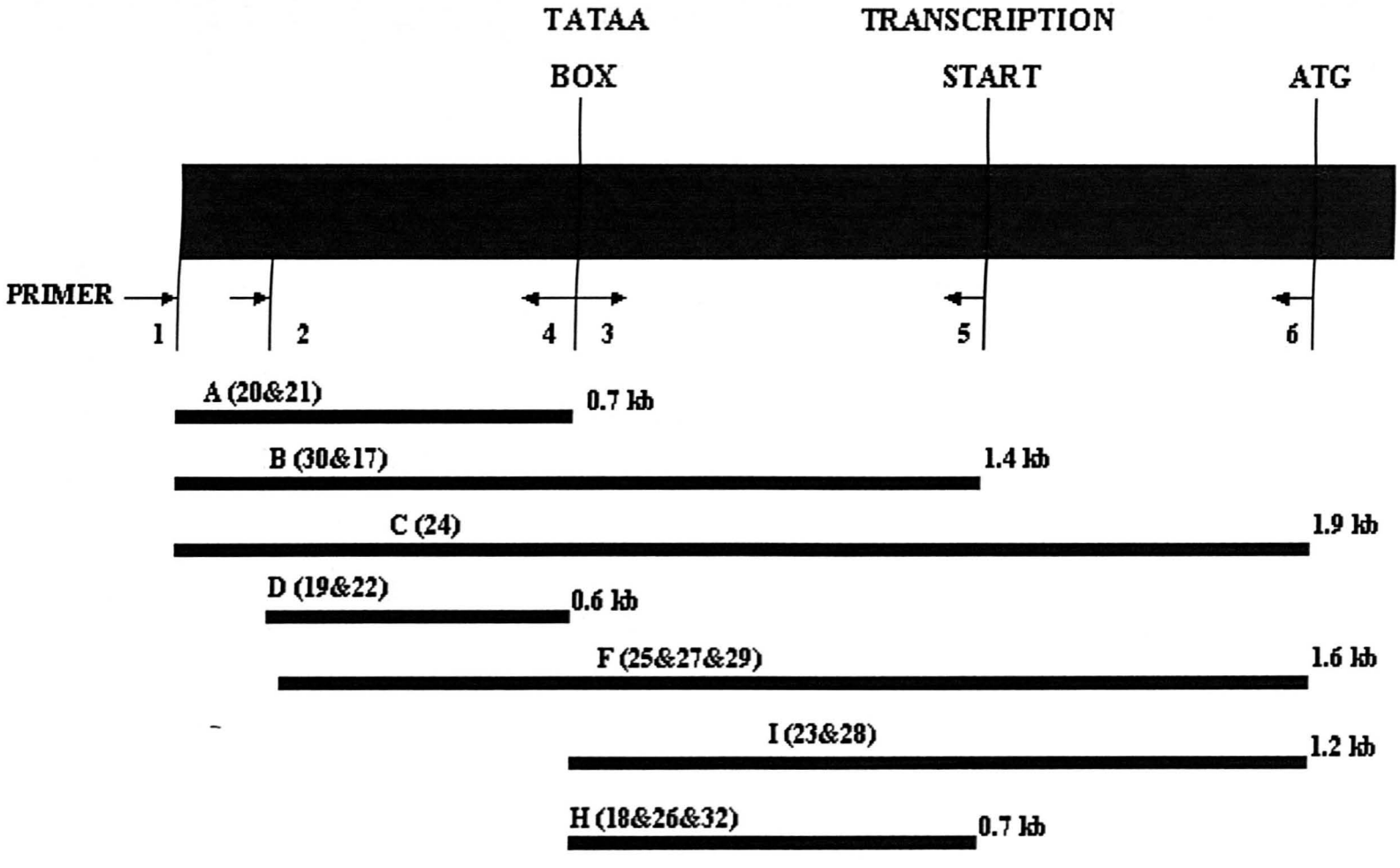


Figure 2.6 Schematic map of the length and region of different *Bvcrk1* promoter fragments.

2.1.18 *Bvcrk1* promoter constructs

Analysis of the genomic clones and comparison with full-length cDNA sequences indicated that the clones contained approximately 1.4 kb sequence upstream of the predicated transcription start site. Analysis of this "promoter" region (using the GCG Findpatterns programme with the tfsites database or individual transcription factor data) for potential transcription factor binding sites revealed that the promoter of *Bvcrk1* did not exhibit what might be considered to be a normal structure. A consensus sequence for transcription initiation, a cap-site and a CAAT box were present close to the transcription start site, as would be expected, but no TATAA box could be found in the same region. A TATAA box was predicted at approximately 700 bp from the transcription start site. Many other transcription factor binding sites were predicted, including a plant G-box, an auxin box, and a site that corresponds to a mammalian Fos binding site. Whether any of these motifs actually bind transcription factors or is involved in modulating *Bvcrk1* expression is as yet unknown.

Analysis of the 5' cRACE products and comparison with the genomic sequence showed that *Bvcrk1* has a long (494 bp) and complicated 5' UTR. The 5' UTR contains a small intron (97 bp) and also has three small open reading frames. 5' UTR in general are known to influence gene expression patterns of a variety of genes in a range of organisms. More specifically, introns are also known to play roles in the regulation of gene expression in some cases.

Two promoter fragments were used in this study. *Bvcrk1-17* (P) contains approximately 1.4 kb of sequence upstream of the predicted transcription start site. *Bvcrk1-26* (P) is a truncated 0.7 kb fragment upstream of the transcription start site (Figure 2.6). These promoter::gusA constructs were introduced into the vector pBECKS$_{400}$.P::GUS (Milan, 2002) and transformed into alfalfa. The sequence of the *Bvcrk1* promoter is shown in Figure 2.5 (the regions of primers to amplify the fragments of the promoter were marked as **1, 2, 3, 4, 5** and **6**). The fragment lengths are shown in Figure 2.6.

1 GATCCCCTTT ACTCTTCCTA CCTTATTTTT GAGTATTTTC CTTAACAAAA 1 →

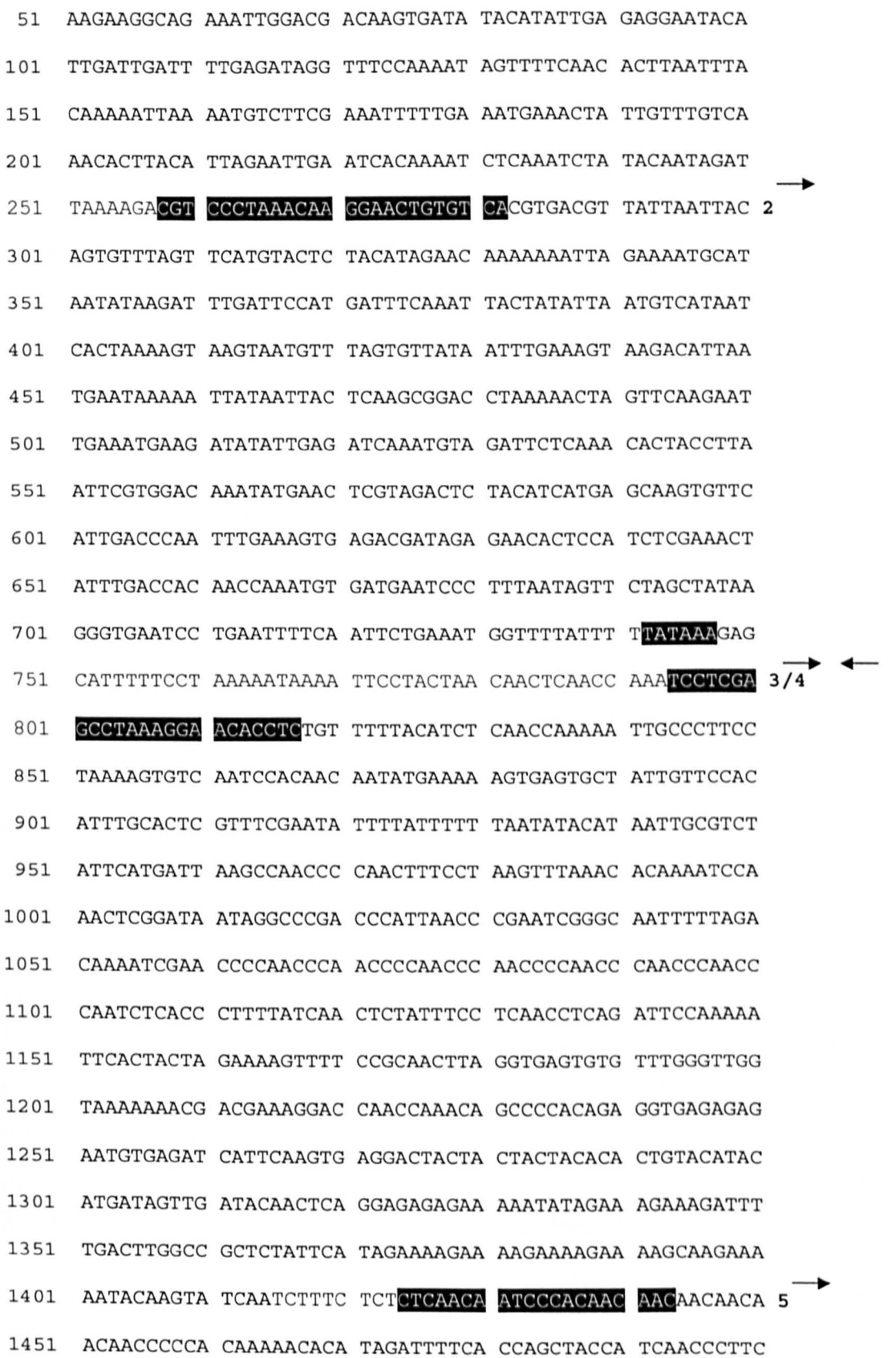

51 AAGAAGGCAG AAATTGGACG ACAAGTGATA TACATATTGA GAGGAATACA
101 TTGATTGATT TTGAGATAGG TTTCCAAAAT AGTTTTCAAC ACTTAATTTA
151 CAAAAATTAA AATGTCTTCG AAATTTTTGA AATGAAACTA TTGTTTGTCA
201 AACACTTACA TTAGAATTGA ATCACAAAAT CTCAAATCTA TACAATAGAT
251 TAAAAGACGT CCCTAAACAA GGAACTGTGT CACGTGACGT TATTAATTAC 2
301 AGTGTTTAGT TCATGTACTC TACATAGAAC AAAAAAATTA GAAAATGCAT
351 AATATAAGAT TTGATTCCAT GATTTCAAAT TACTATATTA ATGTCATAAT
401 CACTAAAAGT AAGTAATGTT TAGTGTTATA ATTTGAAAGT AAGACATTAA
451 TGAATAAAAA TTATAATTAC TCAAGCGGAC CTAAAAACTA GTTCAAGAAT
501 TGAAATGAAG ATATATTGAG ATCAAATGTA GATTCTCAAA CACTACCTTA
551 ATTCGTGGAC AAATATGAAC TCGTAGACTC TACATCATGA GCAAGTGTTC
601 ATTGACCCAA TTTGAAAGTG AGACGATAGA GAACACTCCA TCTCGAAACT
651 ATTTGACCAC AACCAAATGT GATGAATCCC TTTAATAGTT CTAGCTATAA
701 GGGTGAATCC TGAATTTTCA ATTCTGAAAT GGTTTTATTT TTATAAAGAG
751 CATTTTTCCT AAAAATAAAA TTCCTACTAA CAACTCAACC AAATCCTCGA 3/4
801 GCCTAAAGGA ACACCTCTGT TTTTACATCT CAACCAAAAA TTGCCCTTCC
851 TAAAAGTGTC AATCCACAAC AATATGAAAA AGTGAGTGCT ATTGTTCCAC
901 ATTTGCACTC GTTTCGAATA TTTTATTTTT TAATATACAT AATTGCGTCT
951 ATTCATGATT AAGCCAACCC CAACTTTCCT AAGTTTAAAC ACAAAATCCA
1001 AACTCGGATA ATAGGCCCGA CCCATTAACC CGAATCGGGC AATTTTTAGA
1051 CAAAATCGAA CCCCAACCCA ACCCCAACCC AACCCCAACC CAACCCAACC
1101 CAATCTCACC CTTTTATCAA CTCTATTTCC TCAACCTCAG ATTCCAAAAA
1151 TTCACTACTA GAAAAGTTTT CCGCAACTTA GGTGAGTGTG TTTGGGTTGG
1201 TAAAAAAACG ACGAAAGGAC CAACCAAACA GCCCCACAGA GGTGAGAGAG
1251 AATGTGAGAT CATTCAAGTG AGGACTACTA CTACTACACA CTGTACATAC
1301 ATGATAGTTG ATACAACTCA GGAGAGAGAA AAATATAGAA AGAAAGATTT
1351 TGACTTGGCC GCTCTATTCA TAGAAAAGAA AAGAAAAGAA AAGCAAGAAA
1401 AATACAAGTA TCAATCTTTC TCTCTCAACA ATCCCACAAC AACAACAACA 5
1451 ACAACCCCCA CAAAAACACA TAGATTTTCA CCAGCTACCA TCAACCCTTC

Figure 2.5 The nucleotide acid sequence of *Bvcrk1* promoter.

2.2 Materials and Methods

2.2.1 Plant materials for transformation

An autotetroploid line of alfalfa (*Medicago falcata*) 47/1-5, with high capacity for direct somatic embryogenesis was used. Sterile plantlets were grown on MS medium containing 0.8% (w/v) plant tissue culture agar and 1% sucrose, pH 5.5 at 24°C under a 16 hour photoperiod and were subcultured by nodal cutting every 30 days.

2.2.2 *A. tumefaciens* strain and plasmids used for transformation

Agrobacterium tumefaciens strain EHA 101 was used for plant transformation in this chapter (in chapter 4, another *Agrobacterium tumefaciens* strain LBA4404 will be used for HD-Zip gene transformation). A construct of the vector pBECKS400.P-GUS (McCormac *et. al.*, 1998) containing inserts of the promoter region of the cdc2-like protein kinase genes *Bvcrk1* (Beta vulgaris cdc2 related kinase) (Fowler *et. al.*, 1998b) were introduced into strain EHA 101 by electroporation. This plasmid includes the *gusA* gene under the control of the inserted promoter fragment *Bvcrk1-17* (or *Bvcrk1-18*, *Bvcrk1-26*, *Bvcrk1-29*, see figure 2.4 and 2.6) and carries the *nptII* selectable marker conferring kanamycin resistance to transformed plant cells. Strains of EHA 101 harbouring pBECKS400.P-GUS containing inserts of the *Bvcrk1* gene promoter was cultured into 20 mL LB supplemented with spectinomycin at 200 mg L^{-1} in 50 mL sterile screw-capped centrifuge tubes. The tubes were incubated horizontally in an orbital incubator (170 rpm, 28°C) for 2 days, when the culture reached the concentration at approximate OD_{600} =1.0.

2.2.3 Transformation procedure and plant regeneration

Regeneration of transformants via direct somatic embryogenesis from alfalfa leaf explants was delivered by the standard (MSH) system established in the NBI laboratories (Atanassov *et al.*, 1988, 1998; Shao *et al.*, 1997, 1998, 2000). In the standard MSH system, the plant growth regulator combination during a 10-day induction period was 22.6 μM 2,4-D and 4.7 μM kinetin.

Leaves of alfalfa plants were cut into 3-4 mm squares or pieces in MS medium and then transferred into a MS suspension of *A. tumefaciens strain* EHA 101 at cell density that gave an A_{600} of 0.3-0.5. Co-cultivated leaf explants or petiole pieces were incubated on MS medium supplemented with 22.6 μM 2,4-D and 4.7 μM kinetin (MSH) for 3 days at 24°C, then rinsed in water several times, blotted on sterile filter paper and placed on MSH medium that contained 250 mg L^{-1} Timentin or 300 mg L^{-1} cefotaxime. After 7 days, the leaf explants were subcultured on MS medium with both Timentin (or cefotaxime) and 50 mg L^{-1} kanamycin for another 20 days or longer until callus or embryo formation occurred. The calli or embryos were then transferred to plant growth regulator-free MS medium for plantlet formation.

2.2.4 Confirmation of the transgenic plants by histochemical staining

Leaf explants were incubated overnight at 37°C in 0.5 mg mL^{-1} X-gluc solution. (5mg 5-bromo-4-chloro-3-indolyl-ß-D glucuronide was first dissolved in 60 µL dimethylformamide and then made up to 10 mL with 100 mM sodium phosphate buffer pH 7.0).

2.2.5 Plant materials for direct somatic embryogenesis

In this study transgenic alfalfa plant lines containing the *gusA* reporter gene controlled by the *Arabidopsis cdc2a*, *CycB1*;1 promoters and sugar beet *Bvcrk1* promoter fragments were used. An untransformed alfalfa line 47/1-5 was used as control. All of the transgenic lines were derived from the untransformed line 47/1-5. All sterile plants were grown on MS medium and maintained under the same condition as described in section **2.2.1**.

2.2.6 Direct somatic embryogenesis

The direct somatic embryogenic system (with minor modifications) established in the Bulgarian Centre of the NBI was used for all of the somatic embryogenesis investigations in this project (Denchev, 1991a; Shao, 2000, see Chapter 2). Briefly, the system can be described in the following steps: inoculation – washing – induction – embryo development.

Inoculation - Young trifoliate leaves (5-6 mg fresh weight) from sterile subcultured transgenic plants (derived from untransformed plant 47/1-5) containing the *gusA* reporter gene controlled by the *Arabidopsis cdc2a*, *CycB1;*1 promoters and the promoter of sugar beet *crk1* gene (encoding a novel CDK-like kinase) were chopped into small pieces in liquid B_5 medium supplemented with 0.5 g L^{-1} casein hydrolysate, 0.5 g L^{-1} myo-inositol and 3 % (w/v) sucrose (B_50) (Appendix).

Washing – The leaf pieces were washed for 1min in fresh B_5 0 medium.

Induction - After removing liquid B_50 medium, the explants were transferred into 100 mL flasks containing 50 mL B_5IV (Appendix) liquid induction medium which is B_50 modified

with 4 mg L^{-1} 2,4-D, 0.2 mg L^{-1} kinetin, 1 mg L^{-1} adenine and 10 mg L^{-1} glutathione (Appendix). The suspension cultures were maintained on a rotary shaker at 100 rpm under a 16/8-hour photoperiod at 22°C for 18 days.

Embryo development – The B_5IV liquid medium was removed from the explant suspension cultures containing globular embryos and embryos which had already separated from the explants. The explants were washed 2-3 times with fresh $B_5$0 medium, then washed with $B_5$3M (or B5/3M) medium (with 3% [w/v] maltose instead of sucrose, and 2.5% [w/v] PEG6000) (Appendix) and finally transferred to $B_5$3M medium for 30 days.

The same experiment was carried out using untransformed plant 47/1-5 as control.

2.2.7 Histochemical assay for β-D glucuronidase

For histological assays, alfalfa leaf tissue and clusters containing somatic embryos from transgenic plants *cdc2a*, *CycB1;*1 and *crk1-17* suspension cultures were collected at various times during the induction (day 2, 5, 11 and 18), development, maturation and regeneration stages (day 5, 10, 20 and 40). Material from untransgenic plant 47/1-5 suspension culture was used as control. The details of GUS staining were carried out as described in section **2.2.4.**

2.3 Results

2.3.1 Regeneration of *Bvcrk1* promoter transformants from alfalfa leaf explants

Under the standard regeneration conditions, leaf explants were highly embryogenic and very little callus proliferation occurred. Small globular somatic embryos were visible after 8-10 days incubation on basal MS medium and the subsequent 10 days of incubation were characterised by a "wave" of somatic embryogenesis. Small globular somatic embryos occurred along the wounded edges of the explant then throughout the whole of the surface of the explants. Only a small number of embryos were able to grow to green bottle-shaped or cotyledonary somatic embryos. Relatively little callus proliferation occurred and after a

total of 28-35 days in culture, the explants were often completely covered with bottle-shaped or cotyledonary somatic embryos.

Somatic embryos formed singly, in clusters or in polyembryonic structures. Once the green bottle-shaped or cotyledonary somatic embryos formed, the shoots were easily developed and followed by a profusion of abnormal leaves developing. After several rounds of recovery from clusters of abnormal leaves, the trifoliate leaves appeared and led to the formation of normal whole plantlets. Most of the somatic embryos from the early stage failed to develop, some grew into calli, some proliferated to produce secondary embryos, whilst some developed abnormal cotyledons. Further development stopped and these embryos eventually died before reaching the bottle-shaped or cotyledonary stages. One or more plantlets could be obtained from a single leaf explant. The percentage of explants producing kanamycin-resistant green calli/embryos was about 4-6 %. The putative transgenic plants were obtained from transformed leaf explants within 9-15 weeks *via* the MSH system.

2.3.2 Optimisation of the bacterial overgrowth control by antibiotics

In the MSH transformation system, the chopped leaf explants were inoculated with *Agrobacterium* EHA101 strain carrying a transformation vector construct prior to incubation on MSH medium. After 3 days co-cultivation on MSH to allow *Agrobacterium* infection, the inoculated leaf pieces were washed well to remove *Agrobacterium* and then transferred onto MSH medium containing Timentin or cefotaxime (or carbenicillin) which kill *Agrobacterium* cells without harming the plant cells (if the concentration of antibiotics is not too high). The results of plant regeneration showed that the optimal concentration of antibiotics to control *Agrobacterium* overgrowth was Timentin at 200 mg L^{-1}, cefotaxime at 300 mg L^{-1}. Higher concentrations of these antibiotics inhibited the regeneration of plantlets, and even killed the plant cells. Carbenicillin proved not to be an ideal antibiotic to control overgrowth of *Agrobacterium* EHA 101 strain; 100-300 mg L^{-1} carbenicillin was not high enough to control the overgrowth and higher concentrations of carbenicillin resulted in high toxicity to the plant cells, especially in the medium containing both carbenicillin and 2,4-D. The toxicity of combinations of carbenicillin and 2,4-D could be

caused by the auxin-like activity of carbenicillin (Lin *et al.*, 1995), resulting in fewer somatic embryos and hence fewer plants.

2.3.3 Optimisation of the kanamycin selection regime

The effect of selective agents on transgenic plant regeneration should be fully understood to be used most effectively to prevent the growth of untransformed plant materials. Kanamycin is widely used as a selective agent for transformed plants. Kanamycin resistance was a very powerful screening tool for transgenicity in alfalfa (Desgaanes, 1995; Shao *et al.*, 2000). The endogenous resistance was negligible in the regenerative genotypes used in this transformation experiment. However, kanamycin proved to be toxic to plants at high concentrations. Therefore, the concentration of kanamycin becomes a key consideration in the MSH system. Embryos/plants were not obtained using 100 mg L^{-1} kanamycin in the MSH medium (Shao, 2000). Fifty mg L^{-1} kanamycin was normally sufficient for the elimination of untransformed materials. In order to reduce the impact of kanamycin on the regeneration of explants, the addition of kanamycin to the MSH medium during 10-day induction period was delayed. The results showed that regeneration was significantly improved by adding kanamycin on day 5 or day 10 compared to the standard day 3.

The optimal concentration of kanamycin used for selection in this transformation procedure was 50 mg L^{-1}.

2.3.4 Confirmation of *Bvcrk1* promoter construct transgenic plants by assay of *gusA* gene expression

Initially the histochemial GUS assay was employed to determine the success of the transformation. Young leaves from putative transgenic *crk1-17* and *crk1-26* plants obtained from kanamycin selection were chopped and the explants were cultured in B_5IV liquid medium with shaking. After 7-days induction, the cultured plant materials were taken to perform the GUS staining assay. After over night incubation at 37°C, strong blue staining was found in 9 *crk1-17* transformed plants and another one showed weaker blue

staining, but still a significant level of GUS activity. All ten *crk1-26* plants showed significant GUS activity whereas untransformed plant 47/1-5 exhibited negative GUS staining. A variation in GUS activity between samples from the same lines was observed. Histochemical assay of the transgenic plants is shown on Table 2-2.

Table 2-2 Assays of transgenic plants with Bvcrk1 constructs

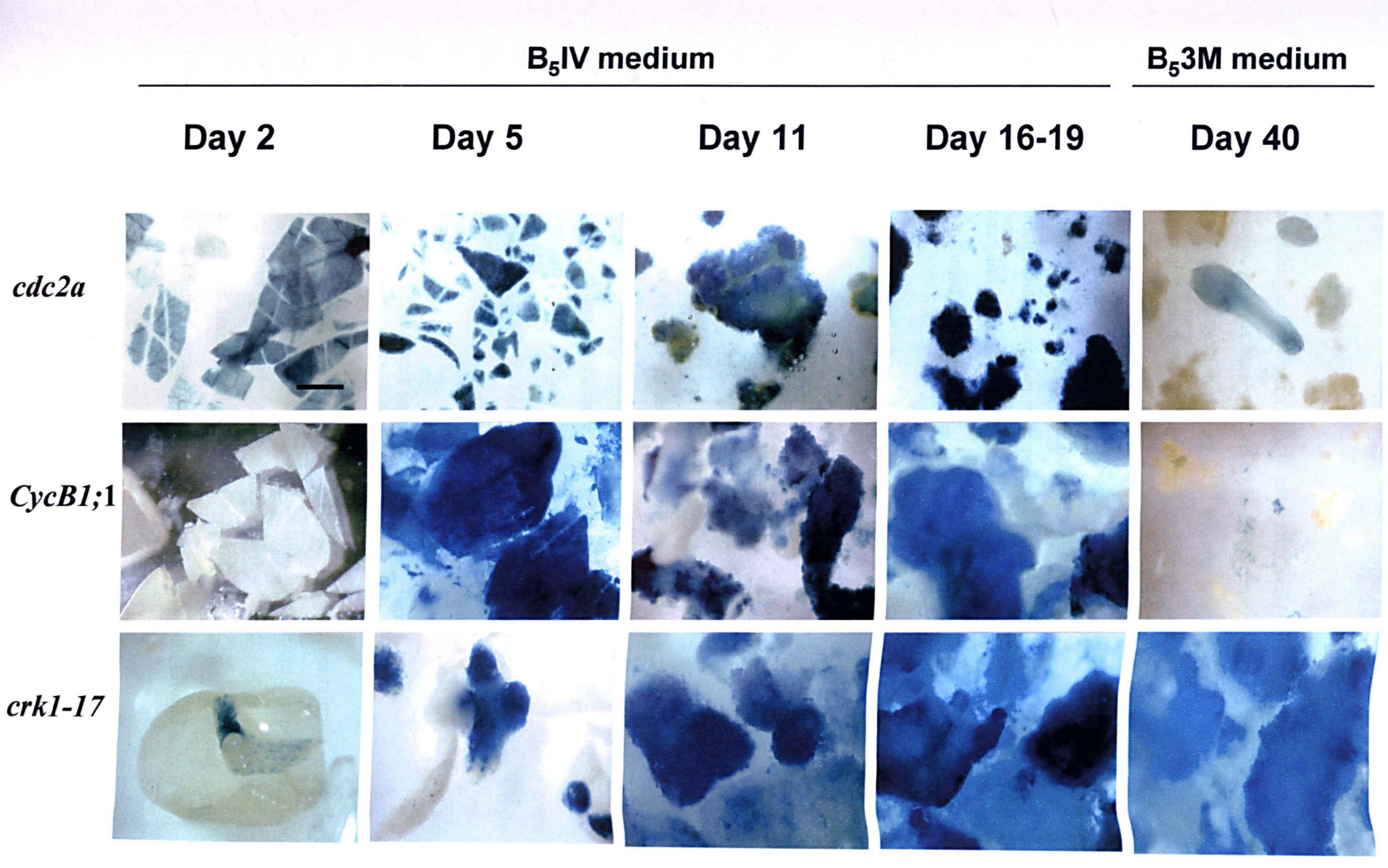
Number	Transgenic Plant	Kanamycin selection	GUS activity (HL)	Regeneration period (weeks)
1	*Bvcrk1-17-1*	+	+++++	9
2	*Bvcrk1-17-2*	+	+++++	10
3	*Bvcrk1-17-3*	+	++++	10
4	*Bvcrk1-17-4*	+	++++	11
5	*Bvcrk1-17-5*	+	++++	12
6	*Bvcrk1-17-6*	+	++++	12
7	*Bvcrk1-17-7*	+	++++	12
8	*Bvcrk1-17-8*	+	++++	13
9	*Bvcrk1-17-9*	+	+++	14
10	*Bvcrk1-17-10*	+	++	15
11	*Bvcrk1-26-1*	+	++++	10
12	*Bvcrk1-26-2*	+	+++	10
13	*Bvcrk1-26-3*	+	++++	10
14	*Bvcrk1-26-4*	+	+++++	11
15	*Bvcrk1-26-5*	+	+++++	12
16	*Bvcrk1-26-6*	+	++++	12
17	*Bvcrk1-26-7*	+	++++	12
18	*Bvcrk1-26-8*	+	++++	12
19	*Bvcrk1-26-9*	+	++++	13
20	*Bvcrk1-26-10*	+	++++	14
21	*Bvcrk1-26-11*	+	++++	15
22	*Bvcrk1-26-12*	+	+++	16
23	47/1-5	–	–	8-10

Note: +, ++, +++, ++++ and +++++ indicate the intensity of positive GUS staining from low to high; –, represents negative GUS staining. For transgenic plants *Bccrk1-17*, samples were taken from 7 days induction with B_5IV for GUS assay; 10 days induction from transgenic plants *Bccrk1-26*).

2.3.5 Expression of cell division cycle genes during induction

Three cell division cycle regulatory gene promoters were used to investigate the activation of cell division cycle progression. The expression patterns of *cdc2a*, *CycB1;*1 and *Bvcrk1-17* construct showed distinctive differences. Following induction, the expression patterns of *cdc2a* and *CycB1;*1 were similar, in that GUS activity was first observed after 1-2 days (for *cdc2a*) or 3 days (for *CycB1;*1), tracing the lines of the leaf vascular elements and subsequently spreading throughout the tissue by 5 days induction (Figure 2.7). By 16~19 days, in the case of *cdc2a* expression, staining was more intense in the globular pro-embryos, but activity could still be detected in the surrounding tissue.

Figure 2.7 Cell cycle gene *cdc2a*, *CycB1;*1 and *Bvcrk1* expression during somatic embryogenesis in alfalfa detected by GUS activity. Time course of induction and development period: 2, 5, 11 and 16~19 days induction with B_5IV medium; after induction period, 40 days with developmental medium $B_5$3M. (bar=500 μm).


B5IV medium
B53M medium
Day 2
Day 5
Day 11
Day 16-19
Day 40
cdc2a
CycB1;1
crk1-17

detected in the surrounding cells (Figure 2.7). In contrast, *CycB1;*1 activity remained high in the pro-embryo cells, but was barely observed in the surrounding cell (Figure 2.7). In the case of *Bvcrk1-17*, the expression pattern GUS activity was first detected by 2 days induction, but the intensity was weak and limited to petiole regions. By 5 days induction, high levels of expression of *Bvcrk1-17* were found, but in localised regions (e.g., leaf veins, petioles), but not in mesophyll tissue. However, a high intensity of GUS staining later developed throughout the whole explant tissue and reached a peak after 9 days induction and was then maintained over the remaining induction period. The expression of *crk1-26* was weaker and considerably delayed, being detectable only after 8 days induction (results not shown).

2.3.6 Expression of cell division cycle genes during development

After induction stage, the explants containing pro-embryos and globular embryos which had separated from the explants were transferred to $B_5$3M developmental medium. The expression of each gene was similar in the early stages (by 5 days in developmental medium) in that GUS activity was spread throughout the whole pro-embryo.

By 11 days, different expression patterns were observed among the three different promoters. The activities of *cdc2a* and *Bvcrk1-17* were still high throughout the whole explant tissue and embryo but in the case of *CycB1;*1, the staining was restricted to meristematic areas. By 40 days in $B_5$3M medium, *cdc2a* and *CycB1;*1 expressions were rare, whereas high expression of *Bvcrk1-17* was still detectable throughout the whole explant tissues (Figure 2.7).

2.3.7 The effect of oryzalin treatment on somatic embryogenesis

To determine the effect of oryzalin treatment, B_5IV medium containing different concentration of oryzalin (0, 2, 15 or 30 μM) was used to induce somatic embryogenesis. Young alfalfa leaves from transgenic plants containing the *cdc2a* reporter gene were chopped and explants were transferred into B_5IV medium containing the cell cycle inhibitor. After 21 days incubation, the suspension cultures were subcultured into $B_5$3M

medium without inhibitor for further somatic embryo development. GUS staining assays were carried out to samples collected on days 1, 2, 3, 7, 12, 14, 16 and 19 in induction medium, and days 2, 5, 14 and 21 in development medium. After only 5 days incubation with $B_5$3M medium, embryos were found in the samples treated with 2 μM oryzalin. By 21 days, embryos were observed from all the treatments. Interestingly, the highest number of embryos came from the 15 μM oryzalin treatment; many of which were larger than normal. Most remarkably, extremely large ("jumbo") embryos were formed in the 30 μM oryzalin treatmed samples. The expression pattern of *cdc2a* detected by GUS activity from each oryzalin treatment was found to be similar. The results are shown in Table 2-3 and Figure 2.8.

Table 2.3. The effect of oryzalin (oryz) treatment on GUS activity and somatic embryogenesis in transgenic alfalfa *cdc2a*At plants.

Days	Medium	GUS Assay			
	B5IV	0 μM (oryz)	2 μM (oryz)	15 μM (oryz)	30 μM (oryz)
1		+	++	++	+
2		++	++	++	+
3		+++	+++	++	+++
7		++++	++++	+++	+++
12		+++	+++	+++	+++
14		+++	+++	+++	++
16		+++	+++	+++	++
19		+++	++	++	+
	B53M				
2		+++	+	+	+
5		++	$+^{E+}$	+	+
14		+++	+	++	++
21		$+++^{E++}$	$+++^{E++}$	$+++^{E++++}$	$+++^{E*+++}$

Note :

+, ++, +++ , ++++ : the level of GUS activity from low to high.E^{+}, E^{++}, E^{+++}, E^{++++}: the number of embryos from low to high. E*: "jumbo" embryo.

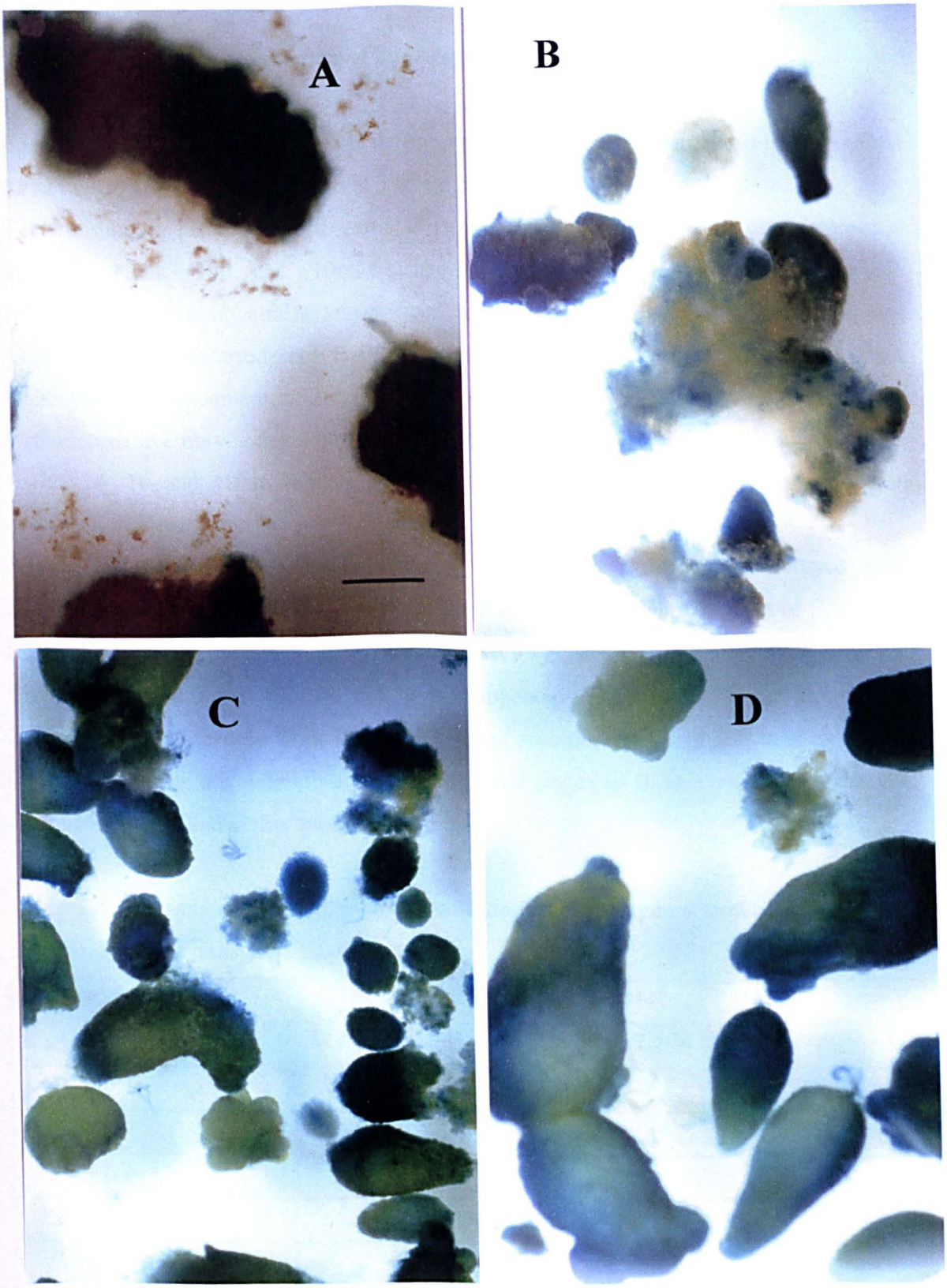


Figure 2.8 Somatic embryos produced from transgenic alfalfa *cdc2a* explants with different concentration of oryzalin treatments. A, B5IV induction (control); B, B5IV+2 μM Oryz, embryos formed early; C, B5IV+15 μM Oryz, highest number of embryos formed; D, B5IV+30 μM Oryz, jumbo embryos formed (bar= 500 μm).

2.3.8 The effective concentration of hydroxyurea treatment

The effect of hydroxyurea (HU) was determined by a similar experiment to that described for oryzalin. The results are shown in Table 2.4.

To determine the effect of hydroxyurea, B_5IV medium containing different concentrations of hydroxyurea (100 μM, 10mM and 100 mM) was used to induce somatic embryogenesis. Young alfalfa leaves from transgenic *cdc2a* plants were chopped and explants were transferred into B_5IV medium containing hydroxyurea. After 20 days incubation, the suspension cultures were transferred to $B_5$3M medium without HU for further somatic embryos development. GUS staining assays were carried out at days 2, 5, 7, 12, 14, 16 and 19 in induction medium and days 6, 13, 20, 27, 35 and 44 in developmental medium.

Among the three different concentration treatments with hydroxyurea, embryos were formed in both 100 μM and 10mM, but not in 100mM treatment (Figure 2.9). The expression pattern of *cdc2a* detected by GUS activity in each HU treatment were different. The treatments at 0 μM, 100 μM and 10 mM appeared to be similar, though the reduced intensity of GUS activity from 10 mM treatment was observed. In 100 mM treatment, GUS activity was not detectable at developmental stage (in $B_5$3M), suggesting that the cells have been killed at this stage.

Table 2.4 Determination of optimal concentration of hydroxyurea treatment in somatic embryogenesis in transgenic alfalfa *cdc2a*.

Day	Medium	GUS Assay			
	B_5IV	0 μM HU	100 μM HU	10 mM HU	100 mM HU
2		+	++	++	+++
5		++++	++++	++++	+++
8		++++	++++	++++	++
14		++++	++++	++++	++
20		++++	++++	++++	+
	$B_5$3M				
6		++++	++++	++	–

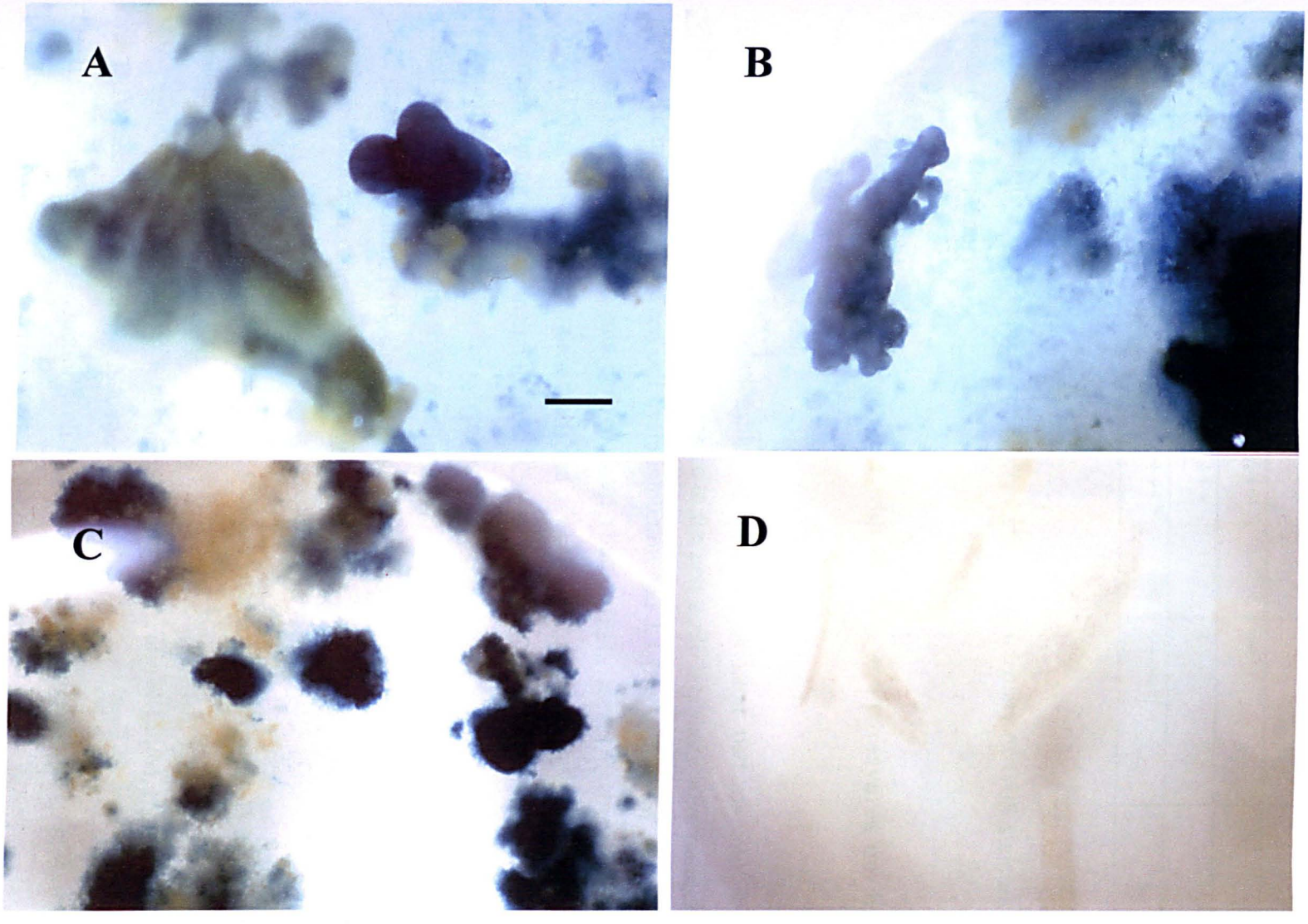


Figure 2.9 Determination of the optimal concentration of hydroxyurea (HU) in transgenic alfalfa *cdc2a* suspension culture. GUS staining after 44 days suspension culture in B53M development medium. A, B5IV induction (control); B, B5IV+100 μM HU induction; C, B5IV+10 mM HU induction; D, B5IV+100 mM HU induction (bar= 500 μm).

13		++++	++++	++	–
20		++++	++++	++	–
27		++++	++++	++	–
35		++++	++++	+++	–
44		++++	$++++^{E+}$	$++++^{E+}$	–

Note:

–: trace or negative GUS expression; +, ++, +++, ++++: intensity of GUS staining from low to high; E+ : embryos.

2.3.9 Cell division pattern of treatment with oryzalin or hydroxyurea during the somatic embryogenesis

In order to determine the relationship between cell division patterns and somatic embryogenesis, shorter periods of exposure to the cell cycle inhibitors were investigated, as was the interaction between cell cycle inhibition and 2,4-D activation of cell division. In these experiments, explants derived from young alfalfa leaves of transgenic plant *CycB1;*1, were used to set up the experiment of somatic embryogenesis with the following treatment during the induction period: *CycB1;1* was chosen in preference to *cdc2a* because it is a specific marker for actively dividing cells.

D: full induction period with B_5IV medium (positive control);

D+Z: induction medium B_5IV containing oryzalin at 15 μM used during induction period;

D+HU: induction medium B_5IV containing hydroxyurea at 10 mM used during induction period;

D→0: after 2 days induction with B_5IV, then transfer to 2,4-D free B_5IV medium for remaining induction period;

D+Z→0: after 2 days induction with B_5IV containing oryzalin at 15 μM, transfer to 2,4-D free B_5IV medium for remaining induction period;

D+HU→ 0: after 2 days induction with B_5IV containing hydroxyurea at 10 mM, transfer to 2,4-D free B_5IV medium for remaining induction period;

0: full induction period with 2,4-D free B_5IV medium (negative control).

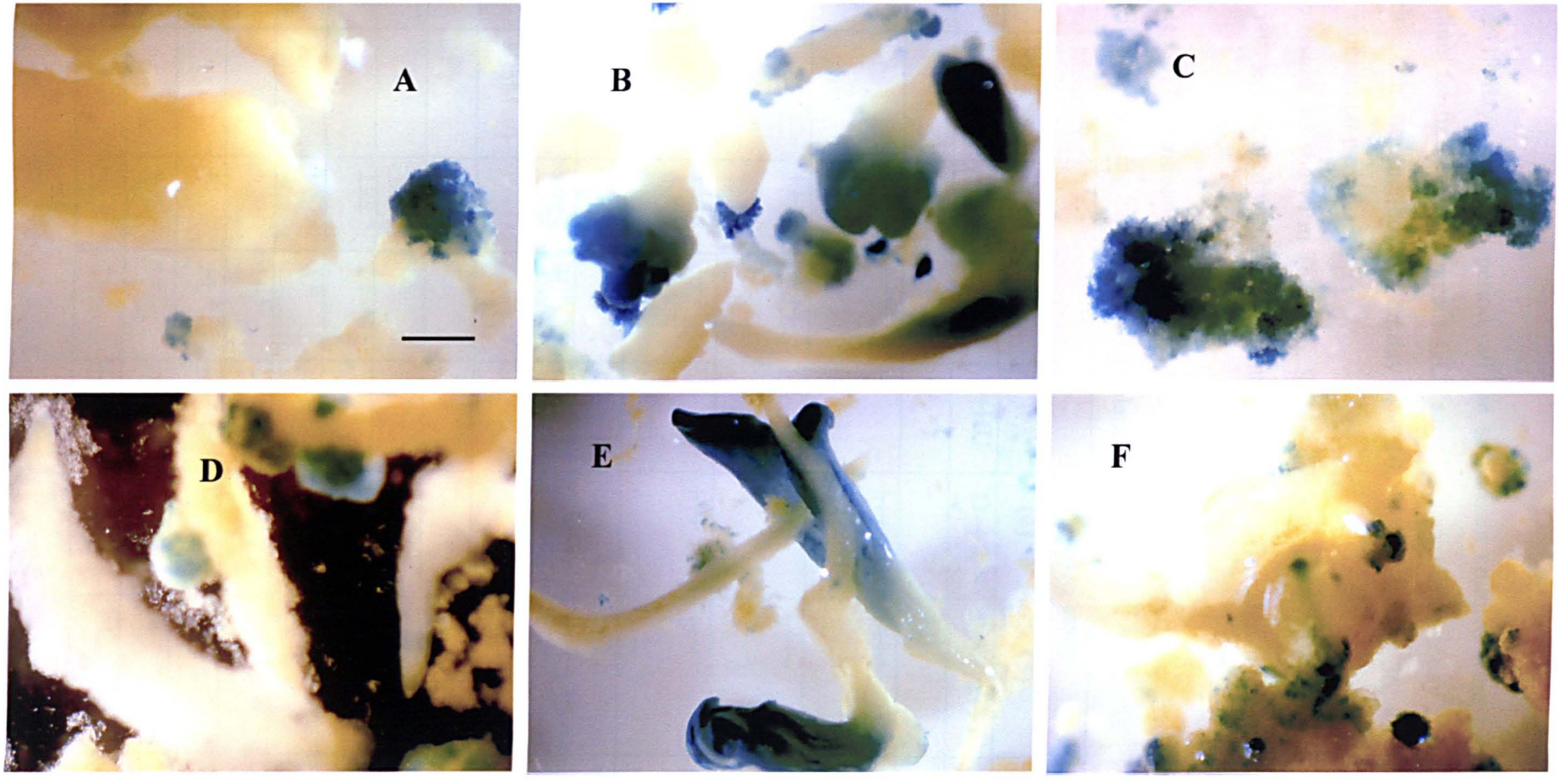


Figure 2.10 Suspension cultures of transgenic alfalfa *CycB1;*1 from 6 different induction treatments with oryzalin (orzy) or hydroxyurea (HU), after 29 days subcultured in development medium $B_5$3M. The induction treatments: A, B_5IV (control); B, B_5IV+oryz; C, B_5IV+HU; D, 2d B_5IV+ 16d B_5IV^{-D}; E, 2d (B_5IV+orzy) + 16d B_5IV^{-D}; F, 2d (B_5IV+HU) + 16d B_5IV^{-D}. Calli and embryos were formed in A; a high number of embryos were formed in B; no calli or embryos found in C; roots formed in D; shoots found in E; roots and calli formed in F (bar = 500 μm).

After 18 days induction period, all the suspension cultures were transferred to $B_5$3M medium for further development. GUS samples were taken on day 2, 5, 7, 11, 14 and 18 during induction period, and on day 4, 7, 10, 14, 17, 23, 29, 36, 43, 52 and 59 during developmental period.

The results are shown in Table 2.5. Suspension cultures from 6 different treatments after 29 days subcultured with development medium $B_5$3M are shown in Figure 2.10.

Table 2.5. Determination of cell division patterns with oryzalin and hydroxyurea treatment by GUS assay using transgenic alfalfa plant *CycB1;*1.

Day	Medium	GUS assay						
	B_5IV	(1)	(2)	(3)	(4)	(5)	(6)	(7)
		D	D+oryz	D+HU	D→0	D+oryz→0	D+HU→0	0
2		–	–	–	–	–	–	–
5		++++	+++	–	+++	++++	+	Trace in veins
7		++++	++++	–	++++	++++	–	Trace in veins
11		++++	+++	–	+++	+++	+	Trace in veins
14		++++	+++	+	++	++	+	–
18		++++	+	+	++	+	+++	–
	$B_5$3M							–
4		+++	+	+	+	+	+	–
7		+++	+	+	+	–	+	–
10		+++	+^{E}	+	+	+	+	–
14		+	+^{E}	+	–	+	++	–
17		+	++^{E}	–	+	+	++$^{E?}$	–
23		+	++^{E}	++	+^{R}	++	+++	–
29		+	++	++	++^{R}	++^{R,E}	++^{R}	–
39		–	+	–	+	++	+	–

43		+	++	+	+	+	+	–
52		+	+	–	+	–	+	–
59		+	–	–	–	+	+++ E?	–

Note: treatment (1), (2), (3), (4), (5), (6), (7) are as described above. E: embryos; R: root. +, indicates positive GUS staining; –, represents negative GUS staining.

In Figure 2.10, calli and embryos were formed in A, (control, B_5IV induction treatment); high number of embryos were formed in B (B_5IV + Z induction treatment); no calli or embryos were found in C (B_5IV + HU induction treatment); roots were formed in D (B_5IV $\rightarrow$0 induction treatment); shoots were formed in E (B_5IV + Z$\rightarrow$0); roots and calli were formed in F (B_5IV + HU$\rightarrow$0).

2.4 Discussion

2.4.1 Optimisation of the MSH regeneration system

The regeneration of plantlets from alfalfa leaf explants *via* direct somatic embryogenesis could be determined by manipulation of the plant growth regulator levels in the medium. In this study, the standard MSH direct somatic embryogenesis system was used to regenerate *Bvcrk1* transgenic plants from transformed alfalfa tissue. The key components involved in the system are 2,4-D at 22.6 μM and kinetin at 4.7 μM which were applied for 10 days induction period. Under this combination and length of induction treatment, plantlets developed from embryos, which were directly formed from leaf explants. Less calli were formed in this system. Similar observations were found in the procedure for generation of *Atcdc2a* (P) and *CycB1;*1(P) transgenic plants (Shao *et al.*, 2000). These results demonstrated that the combination of 22.6 μM 2,4-D and 4.7 μM kinetin for 10 days induction in MSH system is suitable for the transformation with *Bvcrk1-17(Bvcrk1-18, 26* and *29*, data not shown) as well. However, this was later found to be not always the case for transformation of alfalfa with other genes, e.g., in the case of transformation with HD-Zip gene series fragments (see chapter 4) (Zhou *et al.*, 2002, 2003).

2.4.2 The efficient control of bacterial overgrowth during transformation

In *Agrobacterium*-mediated transformation methods, the efficient control of bacterial overgrowth after the co-cultivation period is essential to a successful transformation. The results from these studies have shown that both cefotaxime and Timentin are suitable antibiotics to control EHA101 overgrowth in the MSH transformation system. For cefotaxime, the concentration from 150 to 350 mg L^{-1}, and 150 to 250 for Timentin, is efficient to control bacterial overgrowth (data not shown). Higher concentrations of antibiotics appear to be harmful to the plant cells and result in poor regeneration. Data from the MSH system indicate that the optimal concentration used in alfalfa transformation are; cefotaxime at 300 mg L^{-1} and Timentin at 200 mg L^{-1}. Carbenicillin is not an efficient antibiotic for the choice to control EHA 101 strain.

2.4.3 Confirmation of transgenic status

Bvcrk1-17 transgenic plants were generated from the MSH system with kanamycin selection. A GUS staining assay was used for the further confirmation of transgenic status to the putative transgenic plant candidates. The observations from GUS staining assay showed that all the candidates (10 with construct *Bvcrk1*-17 and 12 with *Bvcrk1*-26) exhibited a high level of positive activity after 7 days (for *Bvcrk1*-17 transgenic plants, or 10 days for *Bvcrk1-26* transgenic plants) induction by 2,4-D (Table 2.2). Similar results were observed from *cdc2*, *CycB1;*1 and *CycA2* transgenic plants (Shao *et al.*, 2000). These results demonstrated that kanamycin at 50 mg L^{-1} is a powerful selection agent during alfalfa transformation.

2.4.4 pBECKS400 is a suitable vector for alfalfa transformation

Binary vector pBECKS400, was developed in the NBI laboratories (McCormac *et. al.*, 1987; 1998). It includes the *gusA* gene under the control of the CaMV35S and carries the *nptII* selectable marker conferring kanamycin resistance to transformed plant cells. Since pBECKS400 was successfully used for the transformation of tobacco (McCormac *et. al.*, 1998), the construct pBECKS400.P-GUS carrying the insert *Bvcrk1-17* promoter has been

introduced into tobacco and carrot (Milan 2002). In the work described as this chapter, pBECKS400.P-GUS construct with *Bvcrk1-17* (P) was introduced into alfalfa plant by *Agrobacterium*-mediated transformation. The whole plants were obtained from the MSH regeneration system within 9-15 weeks (Table 2.1) which showed a similar rate of the growth during regeneration of *Atcdc2a* (P), *CycB1;*1 (P) and *CycA2* (P) transgenic alfalfa plants in a previous study (Shao *et al.*, 2000). The success in the regeneration of *Bvcrk1-17* and *Bvcrk1-26* transgenic plants indicated that pBECKS400 is a suitable vector for alfalfa transformation. With this consideration in mind, pBECKS400/6 was chosen as the vector for the construction of the HD-Zip gene series described in Chapter 4.

2.4.5 cdc2 gene promoter as a molecular marker for the competence of cell division

The induction of direct somatic embryogenesis involves the re-activation of cell division in non-dividing (G0) cells. The re-activation of cell division is closely associated with competence for cell division. During plant development, the pattern of *cdc2a* expression was strongly correlated with the cell proliferation potential. It has been proposed that cdc2a expression may reflect a state of competence to divide (Hemerly *et al.*, 1993). In a previous study of alfalfa, Shao (PhD thesis, 2000 p148) observed that cdc2a expression was not only restricted to dividing cells, but could also be found in non-dividing cells that could be described as competent for division. Similar observations have been found in *Arabidopsis* (Martinez *et al.*, 1992) and soybean (Miao *et al.*, 1993). In this study, *cdc2a* promoter fused *gusA* construct was used as molecular marker to investigate the regulation of cell division and its competence during the induction of somatic embryogenesis. The expression pattern of *cdc2a* revealed by GUS staining assay (Figure 2.7) showed that high levels of *cdc2a* expression reached a peak by 5 days induction and then the high levels of the expression were maintained throughout the remaining period. The results could be explained by the fact that cells can be reactivated and acquire competence to divide after stimulation by appropriate induction. It can be proposed that after induction, cell division competence is necessary for initiation of embryogenesis, at this stage the existing transcriptional and translational profiles are erased or altered in order to allow cells to set a new developmental program.

2.4.6 *Cyc1*At promoter as marker for mitosis during the embryo formation

The *CycB1;*1 promoter construct has been well-studied in several species. To explore the possibility that cyclin transcription plays a role in the developmental regulation of cell division, the spatial and temporal expression of *CycB1;*1 (originally called *cyc1*At) was examined in *Arabidopsis* (Ferreira *et al.,* 1994b). In root and shoot apical meristems and during embryogenesis, *cyc1At* expression is almost exclusively confined to dividing cells. In tobacco protoplasts, an increase in *cyc1*At expression was observed only when cell division was induced. It was proposed that *cyc1*At expression is restricted to dividing cells and is expressed at the transition across the G2→ M-phase boundary (Ferreira *et al.,* 1994b). It is obvious that *cyc1*At expression is tightly coupled with cell division and therefore *cyc1*At could be used as a molecular marker for cell division.

In this study, *cyc1*At (*CycB1;*1) promoter GUS fusion construct was used as molecular marker to investigate the regulation of cell division during the induction of somatic embryogenesis. Results from histochemical GUS assay showed that apart from a slight delay in the first stage at which expression was detected, the expression pattern of *cyc1*At was similar to that of *cdc2a* up to 10 days in induction medium. After 10 days of induction, *cyc1*At expression declined and the expression was only detected in embryonic cells, but not in non-embryonic cells (Figure 2.7). After 18 days in induction medium, the level of *cyc1*At expression continued to decline (Figure 2.7) and histochemical staining indicated that the expression was confined to the globular pro-embryo cells (Figure 2.7). *Cyc1*At is thought to be required for mitosis, so its absence from the non-embryogenic cells suggests that they had exited from the mitotic division cycle. All of the cells were stimulated to divide at the beginning of the induction treatment, but mitosis was not maintained in the non-embryonic cells. This result suggests the possibility that the repression of *Cyc1*At expression may be the mechanism by which mitotic division in non-embryonic cells is switched off.

2.4.7 *Bvcrk1-17* gene expression during somatic embryogenesis in alfalfa

Bvcrk1 expression has been studied in quiescent (G0) sugar beet cell suspension cultures (Fowler *et al.*, 1998). In this system, the regulation of gene expression during the transition from the G0 phase back to the cell division cycle was investigated. The investigation showed that *Bvcdc2* transcripts were present at low levels in quiescent cells (i.e. competent to divide) whereas *Bvcrk1* and *CycB1;*1 transcripts were not detectable. Expression of *Bvcrk1* was induced within 1 h after subculture into fresh medium, whereas *CycB1*;1 transcripts were not detectable until more than 48 h after subculture (Fowler *et al.*, 1998). These observations indicated that the induction of *Bvcrk1* expression occurred very early during the transition from G0 state to the first cell division. In this study, in order to determine the relationship of *Bvcrk1-17* expression to cell division activity and somatic embryogenesis, its expression patterns in alfalfa were compared with the expression patterns of the well-characterised Arabidopsis cell cycle genes, *cdc2a* and *CycB1*;1. Preliminary results from this study indicate that the expression patterns of *Bvcrk1* during the early induction of somatic embryogenesis in alfalfa are compatible with the observations from sugar beet cells quiescent system, given that low level GUS activity could be detected in *cdc2a*, but not in *Bvcrk1-17* and *CycB1*;1 plants by 1 day induction in alfalfa suspension cultures (Figure 2.7). Low levels of expression of *Bvcrk1-17* were detectable after 2 days induction, but *CycB1*;1; was not expressed until 3 days (Figure 2.7). High levels expression of *Bvcrk1-17* were maintained in the suspensions by 40-day subculture with development medium $B_5$3M, but it is not the case of *cdc2a* and *CycB1*;1 (Figure 2.7). In these terms, the expression pattern of *Bvcrk1-17* more closely matched that observed in *cdc2a*, whereas in carrot, *Bvcrk1-17* expression is more closely related to that of *CycB1*;1 (Milan, 2002). In addition, the pattern of early induction of *Bvcrk1* was quite distinct from either *cdc2a* or *CycB1;*1, in that it did not spread from the vascular tissue throughout the mesophyll, but was more localised around the petioles. These facts indicated that the expression pattern of *Bvcrk1-17* in alfalfa may not reflect the same wave of cell division activation reflected by *CycB1;*1 expression.

Bvcrk1-17 construct has shown its several advantages in alfalfa: high level detectable expression of GUS gene and no significantly negative impact found on regeneration and the growth of plant during regeneration of transgenic plants. Similar results were observed

from carrot and tobacco (Milan, 2002). These results suggest that *Bvcrk1* promoter could be considered a suitable candidate as a novel promoter to be used in plant transformation.

2.4.8 The effective concentration of oryzalin and hydroxyurea treatment

In order to determine an effective concentration of cell cycle inhibitors oryzalin and hydroxyurea sufficient to block cell cycle activity but not kill the cells, different concentrations of each cell cycle inhibitor were applied during the induction period in transgenic *cdc2a* plants. It was expected that the higher concentrations of these inhibitors would block cell division during the induction phase and prevent embryo formation. In contrast, the results shown in Table 2.3 and Figure 2.8 show that a significantly greater number of embryos were found in the samples treated with 15 μM oryzalin. Hence, a concentration of 15 μM oryzalin treatment during the induction period obviously promoted somatic embryo formation and therefore was used in later inhibitor combination experiment. With hydroxyurea treatment, embryos were observed after both 100 μM and 10 mM treatments, but not in the 100 mM treatment, which appeared to kill the tissue (Figure 2.9). These data indicate that far from cell division activity being an essential component of the induction phase, the inhibition of cell division during this phase, followed by the release of inhibition when sub-cultured into development medium, is actually more effective for embryo formation.

In addition, an interesting observation was noted from the oryzalin study: that the sizes of embryos formed in the 30 μM treatment were obviously larger than those formed in other treatments (Figure 2.8, D). This observation could also be explained by the cell division block caused by oryzalin during embryo development, since it is possible that the embryogenic competent cells grew larger during this period, rather than dividing rapidly and remaining small. Since oryzalin block in M phase, it is also possible that these large embryos containined endoreduplicated cells. Evidence from histological anatomic and flow cytometric- studies is required for further confirmation.

2.4.9 The regulation of cell division during somatic embryogenesis revealed by the study of cell cycle inhibitors

In this study, two cell cycle inhibitors were used for the investigation of the relationship between cell division patterns and somatic embryogenesis using transgenic *cdc2a* and *CycB1;*1 plants. The expression of GUS gene was detected by histochemical staining assay which represented the levels of *CycB1;*1 and *cdc2a* expression, as markers of cell division and cell division competence, respectively.

In Table 2.5, all of the induction treatments exhibited widely different cell division patterns. In the positive control with a full period of 2,4-D induction treatment (D induction), the highest level of *CycB1;*1 expression detected by GUS assay was observed by 5 days induction, and this level was maintained until 18 days induction, though with a slight decline. After 18 days induction, the suspension cultures were transferred to developmental medium $B_5$3M. A high level of expression of *CycB1;*1 could still be detected by 10 days in $B_5$3M, after then the level of the expression was obviously reduced. Embryos were found on day 59 in $B_5$3M medium. Comparing with full period 2,4-D induction treatment (D induction), the combination with oryzalin (D+Z induction) or hydroxyurea (D+HU) at the most effective concentrations (see section **2.4.8**) showed significantly different cell division patterns. In the combination with oryzalin treatment (D+Z induction), the activity of cell division detected by GUS staining reached a peak by 7 days induction and the high level was maintained until the following 7 days, then the level was much reduced by 18 days induction The reduced level of *CycB1;*1 expression was presented throughout the developmental stage. Embryos were found 10 days after transferring to $B_5$3M medium, which is much earlier than in the case of positive control, and the highest number of embryos were observed. These observations suggest that slowing down the activity of cell division after peak of induction and allowing cell growth and the accumulation of products synthesised in S and G2 phase are helpful in improving somatic embryo formation. In the case of the combination with HU (D+HU induction), the GUS staining results revealed that low levels of GUS activity were detected after 14 days induction and maintained throughout the remaining induction and development period. That neither root nor embryo was formed in this case indicated that activation of cell division during the early stages of the induction is important for root competence and somatic embryogenic competence. Also some components produced from G1/S to G2 may be necessary for embryo formation.

To assess the effects of oryzalin and HU on the induction by 2,4-D, three other treatment regimes were investigated (D→0 (4), D+Z→0 (5), D+HU→0 (6), see table 2.5). After 2 days induction, the suspension cultures were transferred to 2,4-D-free B_5IV medium and maintained until transferred to developmental medium $B_5$3M. The GUS assay results show that cell division patterns from those three induction treatments were different. Similar cell division patterns were found in treatments (4) and (5), where high levels *CycB1;*1 expression were observed by 5 days induction and maintained for about 7 days then the activity of cell division was obviously reduced by 18 days induction. The levels of *CycB1;*1 expression in both (4) and (5) treatments were detectable during the development stage. In the case of treatment (6) (D+HU→0), the GUS assay results revealed that obviously lower and late *CycB1;*1 expression were found during the induction stages compared to treatments (4) and (5). The evidence of root formation from treatment (4), (5) and (6) (Figure 2.10 D, E, F)) suggested that the concentrations of oryzalin and HU used in this experiment do not significantly affect the induction of somatic embryogenesis by 2,4-D. Almost no GUS activity was detected from negative control 2,4-D free B_5IV induction treatment throughout all the induction and developmental stages. In addition, in Figure 2.10, comparison between A and B showed that many more embryos were formed in B; comparison between D and E showed that roots were formed in D, but shoots in E. These observations demonstrated that, once again, oryzalin has positive effects on embryo formation in this study.

Taking all the observations together, it can be proposed that the cell division pattern observed from the induction and in combination of oryzalin treatment may closely reflect the optimal pattern of cell division during the induction of somatic embryogenesis in alfalfa. Furthermore, products produced through G1/S and G2 phase and blocked by oryzalin at G2/M phase may include some important components which are necessary for somatic embryogenesis.

CHAPTER 3 THE ROLE OF 2,4-D IN THE ACTIVATION OF CELL DIVISION AND THE INDUCTION OF SOMATIC EMBRYOGENESIS

3.1 Introduction

3.1.1 2,4-D and the induction of somatic embryogenesis

In higher plants, the differentiation of totipotent embryogenic cells is controlled by a pre-set developmental program, and it results in the formation of terminally differentiated cells. In early embryos, the cells have rapid division cycles and the chromatin becomes transcriptionally active after a variable number of division cycles during embryo development (Dudits *et al.*, 1995). The resetting of the entire ontogenic program following the initiation of somatic embryogenesis requires an essential reprogramming of the gene expression patterns characteristic of the differentiated state back to those that resemble totipotent embryogenic cells (Dudits *et al.*, 1995, Fehér *et al.*, 2003). A considerable amount of evidence from biochemical, physical and structural studies on various embryogenic tissue culture systems has indicated that hormone-induced activation of signal transduction systems plays a central role in these events. Internally transmitted signals trigger substantial changes in chromatin structure, alteration of transcription and induction of a series of cell divisions that leads to the formation of either dedifferentiated callus tissues or somatic embryos. Depending on the complex interaction between cells and external stimuli, two developmental pathways can be controlled by the concentration or duration of auxin treatment. The reprogrammed gene expression profile is reflected by the synthesis of new mRNA molecules after exposing the somatic cells or tissues to hormone treatment under *in vitro* conditions (Dudits, *et al.*, 1995). Among different auxins, 2,4-D is the most commonly applied for somatic embryogenesis induction due to its critical role in the reactivation of the cell cycle and the initiation of embryo formation. Strikingly, auxin (2,4-D) treatment is sufficient to reprogram differentiated somatic cells to regain their totipotency and to achieve a developmental potential similar to that of animal egg cells after fertilization.

A variety of experimental data have indicated a link between exogenously supplied auxin (or combined with cytokinin) and the activation of cell division and subsequent cell

differentiation. For example, a mutation in a putative auxin receptor gene disturbed embryogenesis in *Arabidopsis* (Chen *et al.*, 2001), and in cultures of alfalfa protoplasts, exogenous auxin and cytokinin activated CDKs and cell division (Pasternak *et al.*, 2000). In plant cells treated with a synthetic auxin, such as 2,4-D, the activation of the cell cycle is shown by the initiation of DNA synthesis and subsequent cell division. The primary mechanism of auxin action is related to the binding of the hormone to proteins. Several lines of evidence indicate that membrane-associated auxin-binding sites were located on the endoplasmic reticulum (ER), the tonoplast and the plasmalemma (see review by Palme *et al.*, 1991). Subsequently, auxin-binding proteins (ABP) have been purified from maize shoots (Löbler, *et al.*, 1985; Shimomura, *et al.*, 1986; Napier, *et al.*, 1988; Hesse *et al.*, 1989; Venis, *et al.*, 1987). Some progress has been made in elucidating the biological function of auxin-binding proteins in different cellular localisations by using specific antibodies (Löbler, *et al.*, 1985; Napier, *et al.*, 1988; Hesse *et al.*, 1989; Venis, *et al.*, 1987; Klämbt, *et al.*, 1990; Napier and Venis, 1990).

2,4-D is effective for the induction of somatic embryogenesis in several important model systems (such as carrot, *Arabidopsis* and alfalfa, see Section **1.4.2**) which provide experimental tools for studying the molecular basis of somatic embryogenesis. Direct somatic embryogenesis was observed in alfalfa primary explants such as young leaves, leaf petioles and cotyledons (Denchev *et al.*, 1990, 1991 a, b). In this system, the embryogenic response relies on the ability of the differentiated cells in the primary explants to form embryos directly after wounding and application of auxin (2,4-D) without an apparent process of dedifferentiation. Experimental variation of the induction conditions revealed that cells from different parts of a single leaf explant may have different requirements regarding the duration of the 2,4-D treatment required for embryo formation (Denchev *et al.*, 1991a). An appropriate endogenous phytohormone balance is also considered to be one of the crucial factors influencing the embryogenic potential of explants. The quantity and quality of endogenous hormones (auxin, cytokinins and ABA) was found to be different in different regions of the same plant displaying different embryogenic potential (Centeno *et al.*, 1996; 1997; Hess and Carman, 1998; Choi *et al.*, 1997; Ni, *et al.*, 2001), or even within an explant (Arai *et al.*, 1997; Lo *et al.*, 1997). Only a few somatic cells are sensitive to embryogenesis induction factors and capable of undergoing somatic embryogenesis. In

alfalfa, there are significant differences in the endogenous indole-3-acetic acid (IAA) and abscisic acid (ABA) levels between different embryogenic lines and during the somatic embryogenesis process (Ivanona *et al.*, 1994). The embryogenic lines with the shortest induction phase had the highest IAA and the lowest ABA levels. Moreover, the capacity for direct somatic embryo formation in embryogenic lines declines with increasing age of the leaf and this has also been correlated with a decrease in the endogenous level of IAA in the plantlets from which the explants were excised (Ivanona *et al.*, 1994). These observations have stressed the importance of the developmental stage and the age of the explant for embryogenic capacity, but it is still not clear what the exact trigger for shifting the competent cells into the embryogenic 'determined' state is. The results from extensive experimentation with a variety of embryogenic plant tissue cultures have indicated that exogenously applied auxin (in particular 2,4-D) is one of the key inducers of embryogenic development in somatic plant cells cultured *in vitro* (described in Section **1.4.3**).

The direct somatic embryogenesis system used in this study depends upon the ability of cells within young leaf explants to respond to an induction treatment by the direct formation of somatic embryos without an apparent requirement for an initial stage of disorganized cell proliferation (callus or cell suspension). A range of models can explain this phenomenon. One possibility is that there are cells within the young leaf which are already embryogenically competent and only require an inductive signal to trigger direct embryo formation (Williams and Maheswaran, 1986). At the other extreme, 'direct' systems for somatic embryogenesis do not differ significantly from 'indirect' procedures at the molecular level; both proceed through similar stages of genetic re-programming, albeit at different rates (De Jong *et al.*, 1993; Yeung, 1995; Merkle *et al.*, 1990). These models make quite different predictions about the role of cell division activation in the process. In the first model, the inductive signal acts as a mitogenic trigger which is required only to re-activate cell division in cells which are pre-programmed to divide in a special pattern to form embryos. In the second model, it is assumed that the induction of cell proliferation is required for de-differentiation and that the acquisition of embryogenic competence occurs during or after de-differentiation as in the indirect system. However, both models indicate the central importance of the links between cell division and somatic embryogenesis. Clearly, the role of 2,4-D in this process may be complex and multi-faceted. In the first

model, 2,4-D may be the trigger that activates cell division in embryogenically competent cells, and/or a signal necessary to induce embryogenesis. In the second model, 2,4-D may activate the cell divisions that form de-differentiated cells, but may also be involved in the acquisition of competence and/or the induction of somatic embryogenesis.

In order to investigate the roles of 2,4-D in the induction of direct somatic embryogenesis in alfalfa, it was important to establish methods for monitoring the two processes of cell division and embryo formation independently. In this chapter, *Arabidopsis* cell cycle gene (*cdc2a, CycB1;*1 and *CycA2*) promoters were used as molecular markers to monitor cell division activity, as in Chapter 2. In previous work of the NBI laboratories, these constructs were introduced into alfalfa (Shao *et al.*, 2000), tobacco and carrot (Milan, 2002). Experimental evidence has indicated that *Arabidopsis cdc2a* is a good marker for competence of cell division, while *CycB1;*1 is a suitable marker for mitotic activity. *CycA2* is a typical A type cyclin and exhibits different expression pattern from *cdc2a* and *CycB1;*1 (Fowler *et al.*, 1998).

One approach to separating the roles of 2,4-D in cell division activation and somatic embryogenesis was to examine the effects of the standard alfalfa somatic embryogenesis procedures on transgenic tobacco explants carrying cell cycle reporter constructs. The tobacco was not expected to form embryos under these conditions, and the aim was to determine whether the patterns of cell division activation also differed between the two plant systems.

In parallel, it was necessary to identify potential markers to determine the role of 2,4-D in the induction of somatic embryogenesis. A library of clones expressed in the early stages of the induction phase of the embryogenesis system were isolated by subtractive cloning in the NBI laboratories (Russinova *et al.*, 1998). The aim of this part of the project was to identify potential embryogenesis-related clones from this library and further characterise them, for potential use as markers of embryogenesis.

3.1.2 Genes expressed during the early induction phase of somatic embryogenesis

Subtractive cloning is a powerful method for detecting and isolating gene sequences that are differentially expressed (Happer, 1997). Although there are several different protocols

for subtractive cloning, the basic theory behind subtraction is straightforward. First, two mRNA populations are converted into cDNA: the cDNA that contains specific (differentially expressed) transcripts is referred to as the "tester", and the reference cDNA as the "driver". Tester and driver cDNAs are hybridised, and the hybrid sequences are then removed. Consequently, the remaining unhybridised cDNA represents genes that are expressed in the tester, but are absent from the driver mRNA. Subtractive methods have been widely used to identify differentially expressed genes in plants (Happer, 1997) resulting in various novel genes being isolated. These include genes induced by environmental stimuli (Aguan *et al.*, 1991) and those involved in responses to pathogens (Pautot *et al.*, 1993; Sharma et al., 1993; Wilson *et al.*, 1994; Sato *et al.*, 1995). Subtractive methods have also been used to identify embryo-enhanced genes in the carrot suspension culture system (Zimmerman *et al.*, 1993; Lin *et al.*, 1996). Using this approach, 49 different clones were successfully isolated and all of them were found to be enhanced in globular embryos compared to seedlings (Lin *et al.*, 1996). In our laboratory, a subtractive cDNA library approach was exploited in the alfalfa direct somatic embryogenesis system with the aim of cloning genes related to embryogenic competence. Three different subtractive libraries were constructed from young leaf tissue, after 8 days in induction medium. The driver populations for subtraction of shared sequences were: 1) young non-induced leaf; 2) old (non-embryogenic) non-induced leaf and 3) old induced leaf. The method has shown its efficiency in subtraction and enrichment of cDNA clones, some of which could correspond to the low abundance mRNA present in a small population of embryogenic competent cells. After differential screening of each library, more than 180 clones were isolated and nearly 100 different clones were identified. After DNA sequence characterization, the majority of these clones were identified by sequence homology and categorized by function. The largest group of genes encodes a range of proteins involved in ribosome biosynthesis, translation and post-translational modification. This group of growth-related genes included twenty different ribosomal proteins. Other groups of genes coded for transcription factors and signal transduction components, cytoskeletal proteins, membrane transport proteins, wound and stress-related proteins and proteins involved in electron transport. A full list of identified clones is shown in Table 3.1.

The expression patterns of most of these clones during the induction period was determined by dot blot array hybridisation. It was shown previously in this laboratory that the majority of clones were not expressed in intact leaves, but were induced by the embryogenic induction process. Most genes showed a peak of expression between 5 and 10 days in induction medium. In this chapter, further analyses of the expression patterns of 100 clones by dot blot array hybridization are described in relation to the involvement of 2,4-D in the induction of these genes.

In addition, a small number of genes were selected on the bases of possible involvement of regulatory elements for further characterisation. Full length gene sequences were obtained by 5' end and 3' end RACE extension of the original subtractive clone sequence. Sequencing data from the full length genes make the possibility of protein function prediction more certain. The subsequent characterisation of this collection of clones could ultimately generate a collection of molecular markers for cell type and/or developmental stages during embryogenesis (Zimmerman *et al.*, 1993).

3.2 Materials and Methods

3.2.1 Plant Materials

The untransformed alfalfa (*Medicago falcata*) plant line 47/1-5 (Denchev *et al.*, 1991), transgenic alfalfa lines containing *Arabidopsis cdc2a, CycA2* and *CycB1;*1 promoter::*gusA* constructs (both derived from 47/1-5) (Shao *et al.*, 2000) were used in this study. Transgenic tobacco plants containing *Arabidopsis cdc2a, CycB1;*1 and *CycA2* promoter constructs (obtained from Professor D Inzé, VIB, University of Gent) were obtained by transformation of *Nicotiana tabacum* variety Xanthi (Milan, 2002). Sterile plantlets of both alfalfa and tobacco were maintained in the conditions as described in section **2.2.1.**

3.2.2 The induction of somatic embryogenesis in alfalfa

Young trifoliate leaves from either non-transgenic plants 47/1-5 or from transgenic plants *cdc2a* and *CycB1;*1 (as in section **2.2.6**) were removed, using scissors. Embryo induction

was carried out as in **2.2.6.** The plant materials were chopped to small pieces in B_50 medium (Appendix) using the same method as described in section **2.2.6** and the explants were transferred into a 100 mL flask containing B_5IV induction medium. The explants cultured in 2,4-D free B_5IV induction medium were set up as controls. The cultures were maintained on a rotary shaker under the condition described in section **2.2.6.** After an 18-day induction period, the suspension culture material was transferred into B_53M embryo development medium and grown for several more weeks.

3.2.3 The procedures for the induction of somatic embryogenesis in alfalfa applied to tobacco

The first or second apical leaves from transgenic tobacco plant *cdc2a, CycB1;*1 and *CycA2* were harvested and cut into small size pieces in B_50 medium. The explants were then treated as described for the alfalfa direct somatic embryogenesis procedure (section **2.2.6**).

3.2.4 Histochemical assay for β-glucuronidase activity

The alfalfa, tobacco leaf tissue and suspension cultures were collected at different time points during the incubation with B_5IV induction medium and B_53M development medium. The collected samples from the suspension cultures were used immediately for histochemical assays for β- glucuronidase activity as described in section **2.2.4.**

3.2.5. Duration of 2,4-D treatment and GUS staining assay

Young trifoliate leaves from transgenic alfalfa *cdc2* plants were chopped into small pieces in B_50 medium. After washing, the cut material was transferred into B_5IV induction medium. After, 2, 10 and 60 min, the material was washed using B_50 twice and then transferred into 2,4-D free B_5IV medium (B_5IV^{-D}) for 16-day induction. After a 16-day induction period, the material was transferred into B_53M medium for further embryo development. The control material had a full 16-day induction in B_5IV (positive control)

or in B_5IV^{-D} (negative control) medium followed by 22 days in $B_5$3M development medium. The GUS staining assay was conducted on days 1, 2, 3, 5, 7, 12, 16 of the induction period, and days 5, 7, 12, 17, 22 of the development phase.

3.2.6 Plant material for dot blot array hybridisation probes

Chopped leaf fragments of transgenic plant *c dc2a* were inoculated in B_5IV for varying lengths of time (2, 10, 60 min, as described in Section **3.2.5**), then transferred into B_5IV medium without 2,4-D as described in section **2.2.6**. A 7-day full period of B_5IV medium induction sample was conducted in parallel. After a total of 7 days induction period, the plant materials from suspension culture were collected, washed with sterile distilled water and immediately frozen in liquid nitrogen. For the zero time sample, young leaves were chopped quickly in B_50 medium, then frozen in liquid nitrogen after removing the liquid medium. The frozen plant materials were stored at –70°C before extraction of mRNA.

3.2.7 Preparation of DNA samples by PCR

All of the subtractive cDNA was cloned into pT-Adv Vector (Clontech) and transformed into TOP 10 F' *E.coli* (Clontech). The plasmids were used as templates to carry out PCR, using generic primers specific to the multi-cloning site of the vector, one on each side of insert. The subsequent subtractive cDNA clones PCR products were used for the dot blot assay.

3.2.8 Dot blot array hybridisation

Before spotting the subtractive clones PCR products onto a nylon membrane, 10 μL sample from each clone PCR products of 100 clones was gently mixed with 10 μL buffer (10 x SSC, 800 mM NaOH). The 20 μL mixture of each sample was spotted onto BrightStar- Plus TM Nylon Membranes (Ambion) and crosslinked by baking in an oven at 80°C for 15-30 min.

3.2.9 mRNA isolation

All of the glassware and plastics were kept in an RNase-free condition. All reagents used were of analytical grade and RNase-free. All the solutions involved in this experiment were made with sterile double distilled water without DEPC treatment.

mRNA was isolated directly from alfalfa leaf tissue using the Dynabeads Oligo $(dT)_{25}$ Biomagnetic Separation System according to the instruction provided in the Dynal Technical Handbook (Dynal A. S., Norway) with the modifications described below. Before the extraction, plant material was ground into fine powder in liquid nitrogen using a pre-chilled mortar and pestle. Approximately 100 mg frozen powder was transferred to each Eppendorf tube containing 1.0 mL Lysis/Binding buffer (100 mM Tris-HCl pH 7.5, 500 mM LiCl, 10 mM EDTA pH 8.0, 1.0% SDS and 5 mM DTT), 5 tubes from each treatment. The frozen powder was homogenized until complete lysis was obtained (approximately 1-2 minutes), and then direct mRNA isolation was carried out exactly following the description in the protocol supplied. After the first round mRNA isolation, Dynabeads Oligo $(dT)_{25}$ were regenerated as described in the Dynal Technical handbook and reused four more times. Using the same $(dT)_{25}$ (250 μL) for the same time point enabled one tube of Dynabeads Oligo $(dT)_{25}$ to be used for each of the five aliquots of one sample. After elution of mRNA from Dynabeads Oligo $(dT)_{25}$, mRNA from the same treatment was transferred to the same RNase-free Eppendorf tube and stored at –70°C.

3.2.10 Labeling of the mRNA probes

0.5 μg of mRNA from each treatment was labeled in a 10 μL reaction with BrightStarTM Psoralen-Biotin Nonisotopic Labeling kit (Ambion) according to the manufacturer's instructions. After labeling, the probe concentration and the efficency of the labeling reactions was estimated by dot blot and immunodetection, 500 pg, 50 pg, 5 pg, 500 fg and 50 fg of labeled mRNA from each treatment and 100 pg, 10 pg, 1 pg, 100 fg and 10 fg of Psoralen-Biotinylated Control DNA were spotted onto BrightStar-PlusTM Nylon membrane separately and crosslinked by baking at 80°C for 15-30 minutes. Detection was

done using BrightStarTM BioDetecTM Nonisotopic Detection Kit (Ambion) according to the instruction of the supplier.

3.2.11 Dot blot hybridization

Dot blot hybridizations were performed using NorthernMaxTM Complete Northern Blotting Kit (Ambion) according to the instruction manual of the supplier. Prehybridisation and hybridization were carried out using plastic hybridization bags in a 42°C water bath. Prehybridisation should be at least 30 minutes and then hybridization was maintained overnight (8-6 hours). The optimal probe concentration, used in all experiments, was 0.5 µg BrightStarTM Psoralen- Biotin non-isotopic labeled mRNA. After completing the hybridization, the stringency washing was carried out as described in the manufacturer's instructions. High stringency washing was carried out once for 15 minutes at 42°C with agitation and then for 30 minutes under the same conditions. Signal detection was done using BrightStarTM BioDetecTM Nonisotopic Detection Kit (Ambion) exactly following the instruction described in the protocol supplied.

3.2.12 Data analysis by IT processing

The results from autoradiographs were analysed by scanning the patterns into a PC using a HP ScanJet 4C scanner and analysed using the SigmaScan program (Jandel). Intensity reading on the greyscale were converted to an arbitrary 0-100 intensity scale, where 0 = the background intensity of the film (lightest) and 100 = 0 on the grayscale (darkest).

3.2.13. Subcloning of RACE products derived from original subtractive clones

3.2.13.1 Transformation of RACE fragment insert ligated with pGEM-T Easy vector into XL 10-Gold Kan ultracompetent cells

3.2.13.1.1 Ligation of RACE fragments with pGEM-T easy vector

The purified RACE fragments from the GeneClean procedure were ligated into the pGEM-T Easy vector, respectively. Each ligation was set up in a volume of 10 μL including 5 μL 2X rapid ligation buffer, 1 μL pGEM-T easy vector, 2 μL diluted (5x dilution) GeneClean Kit III (BIO 101: Anachem, Beds, UK) purified PCR products, 1 μL ligase and 1 μL H_2O. The ligation was incubated at 4°C overnight.

3.2.13.1.2 The transformation of the ligations (vector with insert) into XL10- Gold Cell

The tube of XL 10-Gold Kan ultracompentent cells (supplied by Stratagent) was thawed on ice. After gently mixing by hand, an aliquot of 40 μL of the cells was transferred into a chilled, 15 mL, Falcon 2059 polypropylene tube. Then 1.6 μL of the XL 10-Gold ß-mercaptoethanol provided with the kit was added into the tube. The contents of the tube was swirled gently and incubated on ice for 10 minutes with gently swirling every 2 minutes. Two μl of the ligating reaction was mixed with the transformation reaction then swirled gently. The tubes were incubated on ice for 30 minutes, then given a heat pulse in a 42°C water bath for 30 seconds. The tubes were incubated on ice for 2 minutes, then 0.45 ml of preheated (42°C) NZY+ broth was added to each tube, and incubated at 37°C for 1 hour with shaking at 225–250 rpm. 150 μL of each experimental transformation reaction was plated onto LB-ampicillin containing IPTG and X-gal agar plates using a sterile spreader. The plates were incubated at 37°C overnight.

3.2.13.2 Colonies selection and examination

Five pure single white colonies were chosen from each transformation and inoculated into tubes containing LB-ampicillin liquid medium individually. The suspension cultures were incubated at 37°C overnight with shaking (~150rpm).

The above suspension cultures were processed for plasmid DNA isolation using the Wizard *Plus* Minipreps DNA Purification Kit. The procedures were carried out according to the protocol instruction included with the Kit.

The concentration of plasmid DNA samples was determined by measuring the A_{260} in a spectrophotometer.

3.2.13.3 Confirmation of the transformation by restriction double digestion

One μg pGEM-T Easy plasmid DNA from each sample was used to carried out a restriction enzyme double digestion with *Sac*I and *Sac*II to confirm the transformation. The restriction enzyme digestion was conducted in a total volume of 20 μL including 2μL MC-buffer, 0.2μL BSA, 0.5μL *Sac*I and 0.5μL *Sac*II (both SacI and *Sac*II supplied by Promega). The digest reactions were incubated at 37°C overnight, followed by gel electrophoresis. The successful transformations were determined by analysis of double digestion products based on the results obtained from gel electrophoresis.

3.2.13.4 DNA sequencing

Two μg plasmid DNA sample containing the relevant insert (confirmed by double digestion) was prepared using Wizard *Plus* Minipreps plasmid DNA purification Kit (Promega) or Qiagen Miniprep plasmid DNA purification Kit (Qiagen) and sent to Oxford DNA Sequencing Service for sequencing.

3.2.14 Sequence analysis by database homology searching

Sequence data returned from automated sequencing were checked for ambiguities. Where possible, ambiguities were resolved by analysis of the sequencing trace using the Chromas program. Sequences of RACE products were aligned with the original subtracted cDNA sequence using the GeneDoc sequence alignment program. The consensus sequence from this alignment was used to search for homologous sequences using either the TBLASTX protocol directly, or the BLASTP protocol with the translated protein sequence.

3.3 Results

3.3.1 Histological changes following the induction of somatic embryogenesis in alfalfa

Several histological changes were observed during the induction process of somatic embryogenesis in suspension culture of chopped leaf explant pieces in liquid medium containing 2,4-D and kinetin. After 5-7 days induction, globular clusters of small cells presented within the explant tissue (Figure 2.7). After 10 days, there was a marked swelling of the leaf pieces resulting in a swollen, spongy appearance. (Figure 2.7) and enlarged cells were found surrounding globular pro-embryo structures (Figure 2.7). Within 15 days, the enlarged cells started to separate and globular pro-embryos were released from the surrounding tissue into the liquid medium (Figure 2.7). No callus-like material was formed from this system. The suspension culture became very thick with released cells and cell clusters after 15 days induction. After the completion of the induction stage of somatic embryogenesis, the pro-embryos were collected and transferred into a development medium $B_5$3M containing PEG but lacking several components of induction medium (2,4-D and kinetin free), of which 2,4-D is the most significant. The formation of somatic embryos developed through the heart and torpedo stages. Different stages of embryo development were observed since the process of embryo induction was not synchronized (Figure 2.7, *cdc2a*). Subsequent subculture on to solid MS medium led to the formation of whole plantlets.

3.3.2 Histological changes pattern following the induction treatment by 2,4-D applied in tobacco

The pattern of histological changes following the induction treatment to tobacco leaf explants as applied in the alfalfa somatic embryogenesis procedure was characterized. After 5-7 days induction in B_5IV medium containing 2,4-D and kinetin, the explants became swollen and spongy (Figure 3.1, 3.3 and 3.5). Cells separated from the explant and released into the medium, where enlarged and elongated cells were observed within 21 days of incubation (Figure 3.1, D). Small round cells were not found and, subsequently, embryos were not produced throughout the whole procedure. Remarkably elongated cells were found after the completion of the induction stage (Figure 3.2). Shoots

were observed from *CycB1;*1 explants with B_5IV^{-D} (2,4-D free) induction treatment after 43 days subcultured with $B_5$3M medium (Figure 3.4).

3.3.3 Histochemical assay of activation of cell division genes during the induction

3.3.3.1 Expression of cell division cycle genes in alfalfa

Two constructs containing cell division cycle regulatory gene promoters from *Arabidopsis*, *cdc2a* and *CycB1;*1 linked to *gusA* were used to investigate the activation of cell division during somatic embryogenesis in alfalfa. A distinctive difference was found between the expression patterns of *cdc2a* and *CycB1;*1. Initially during the induction period, the expression pattern of each gene was similar, in that GUS activity was first observed after 1-2 days (*cdc2a*) or 3 days (in *CycB1;*1) tracing the lines of the leaf vascular elements and subsequently spreading throughout the tissue by 5 days (Figure 2.7). In the case of *cdc2a*, staining was still observed throughout the tissue after 19 days; it was more intense in the globular pro-embryos, but activity could still be observed in the surrounding cells (Figure 2.7). In contrast, *CycB1;*1 activity remained at a high level in the pro-embryo cells, but was rarely detectable in the surrounding cells by 18 days (Figure 2.7). After transfer of the material to the $B_5$3M development medium, the expression pattern of the two genes was similar in the early stages (5 days in development medium) in that GUS activity was spread throughout the whole embryo. By 14 days, different expression patterns were observed in the cultures derived from plants containing the two different promoter constructs. The activity of the *cdc2a* promoter was still high throughout the whole embryo, but in the case of *CycB1;*1, the staining was restricted to meristematic areas. The activity of *cdc2* remained high in the developing embryos in 61-day-old suspension culture maintained in development medium $B_5$3M. GUS staining assay of *cdc2a* and *CycB1;*1 expression in alfalfa during somatic embryogenesis are shown in Table 3.2.

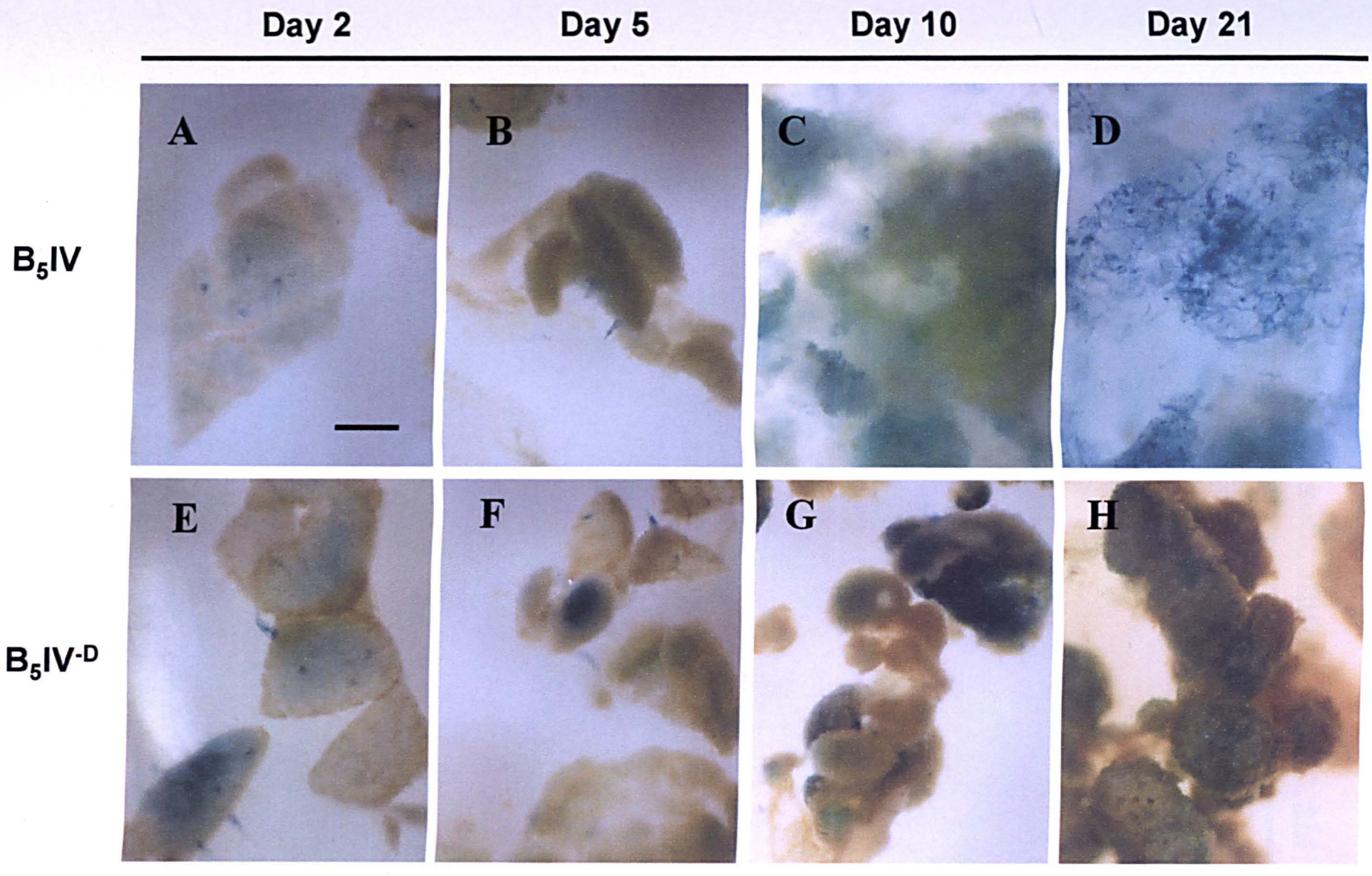


Figure 3.1 The expression of *cdc2a* in transgenic tobacco detected by GUS assay during the induction procedure used in the alfalfa direct somatic embryogenesis system. A-D, induction with B_5IV medium; E-H, induction with B_5IV^{-D} medium. Elongated cells were produceed after 21 days induction with B_5IV (D). (bar = 700 μm).

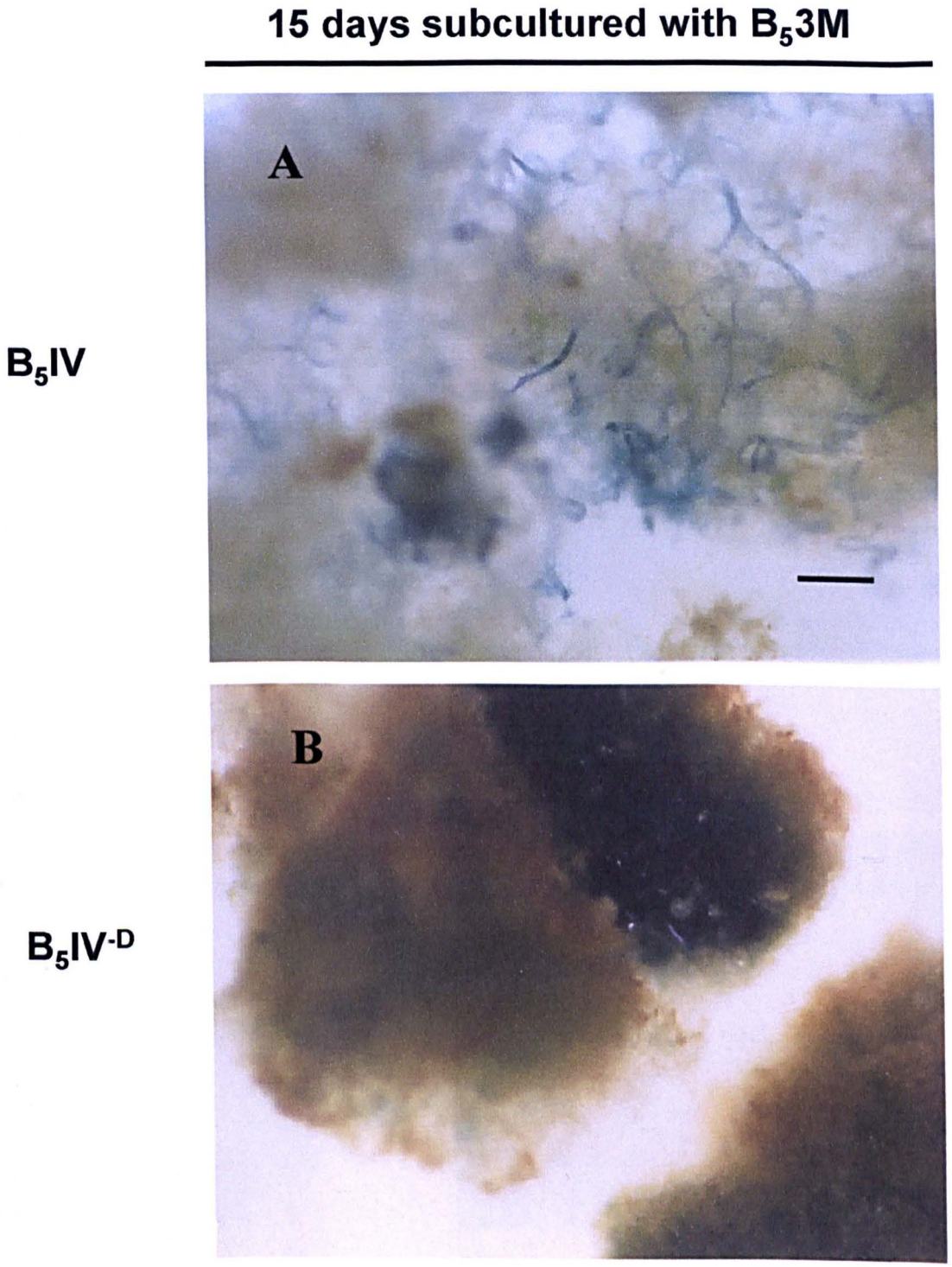


Figure 3.2 GUS assay of explant suspension cultures from transgenic tobacco *cdc2a* after 15 days subcultured in B5/3M development medium. A: induction period with B_5IV (containing 2,4-D) medium; B: induction period with B_5IV^{-D} (2,4-D free) medium. Arrows indicate the elongated cells (bar = 700μm).

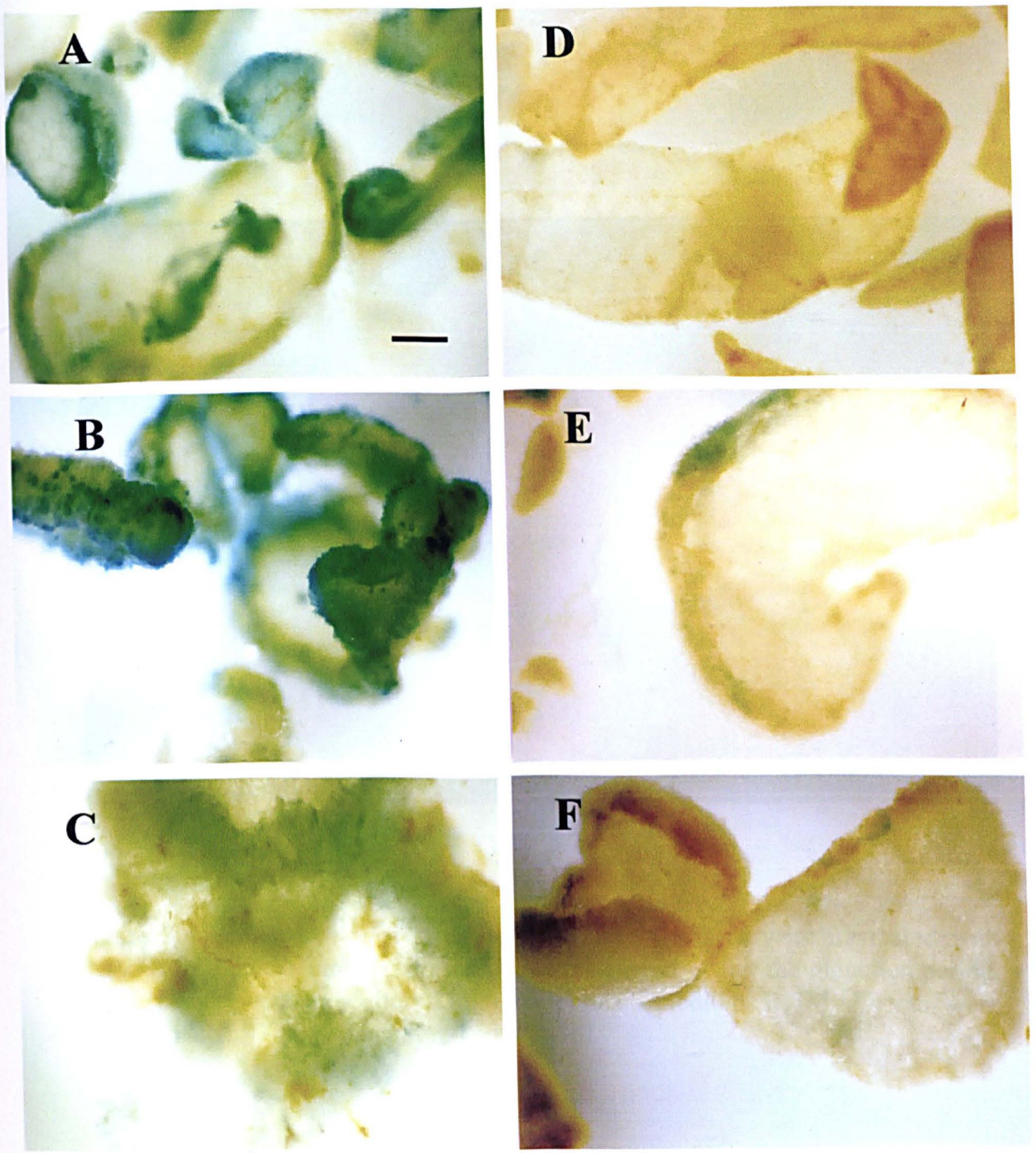


Figure 3.3 Expression of *CycB1;*1 in transgenic tobacco during the induction period in 2,4-D (B_5IV) detected by GUS activity. A-C, induction period with B_5IV medium; D-F, induction period with B_5IV^{-D} (2,4-D free) medium. A, D: 2 days induction; B, E: 5 days induction; C-F: 10 days induction (bar=1 mm).

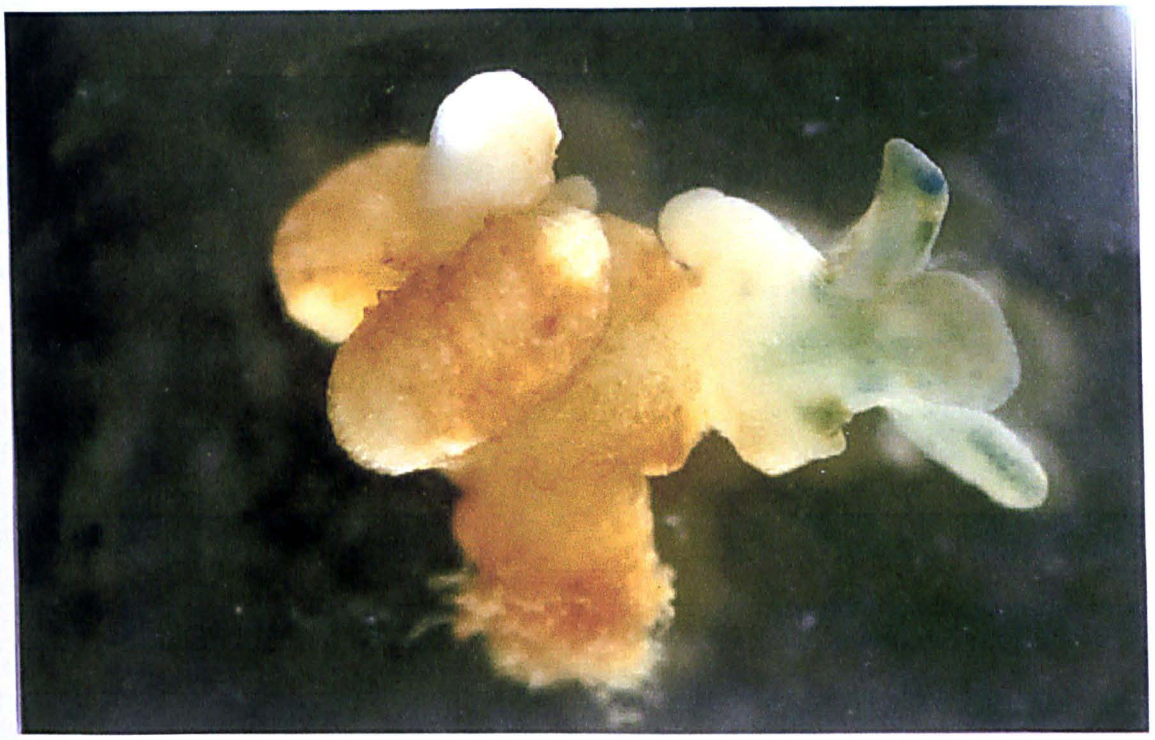


Figure 3.4 Leaf explants of transgenic tobacco *CycB1;*1 were incubated with B_5IV^{-D} (2,4-D free) medium during induction period. After the induction period, the explants were subcultured in development medium $B_5$3M. Shoots were observed after 43 days subcultured with $B_5$3M.

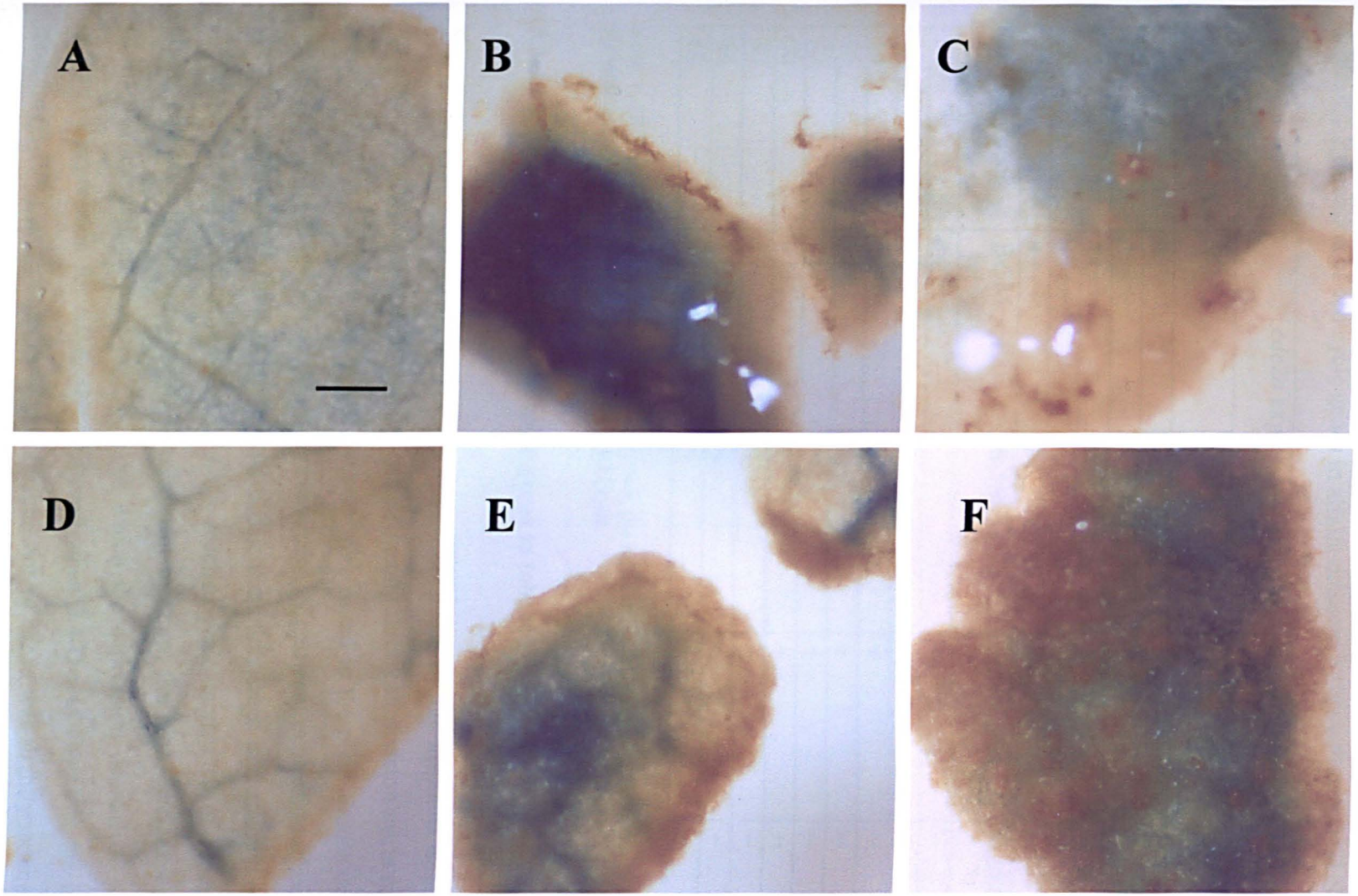


Figure 3.5 *CycA2* expression in transgenic tobacco during the induction period in B_5IV (containing 2,4-D) or B_5IV^{-D} (2,4-D free) medium detected by GUS activity. A-C, induction period with B_5IV medium; D-F, induction period with B_5IV^{-D}. A, D: 2 days induction; B, E: 5 days induction; C, F: 10 days induction (bar= 1 mm)

Table 3.2 Expression of cell cycle genes in alfalfa detected by histochemical GUS assay

Day	*cdc2a* expression		*CycB1;*1 expression	
Induction stage	B_5IV	B_5IV^{-D}	B_5IV	B_5IV^{-D}
2	+	−	−	−
5	++++	−	++++	trace in veins
7	++++	±	++++	trace in veins
14	++++	−	++++	−
18	++++	±	++++	−
Development stage	B_53M	B_53M	B_53M	B_53M
5	++++		+++	−
10	++++	±	+++	−
14	+++	±	+	−
18	+++	±	+	−
23	+++	−	+	−
30	+++	−	+	−
37	++	−	−	−
44	++(E)	−	+	−
61	++(E+)	−	+(E)	−

Note: +, GUS staining positive; +, ++, +++ and ++++, the intensity of GUS staining from low to high; −, GUS staining negative. E, embryos; E+, high number embryos.

3.3.3.2 Expression of cell division cycle genes in tobacco

The activation of cell division induced in tobacco tissue by the standard alfalfa direct somatic embryogenesis system was investigated using three cell division cycle regulatory gene promoters. Different expression patterns were found in the plant tissues containing the *cdc2a, CycB1;*1 and *CycA2* promoter constructs. Significant expression of *cdc2a* was observed from day 3 throughout the remaining induction stage and the whole of the development stage. The expression of GUS reached peak on day 10 of the induction phase, and after transferring to the development medium, the high level expression of *cdc2a* was maintained until day 18, after which the expression decreased. A similar pattern of expression for *cdc2a* was observed when 2,4-D was absent in the induction medium (B_5IV^{-D}), except that the level of expression of *cdc2a* was slightly reduced. In the case of *CycB1;*1, the expression pattern was similar to *cdc2a* during the induction stage.

However, very little expression of *CycB1;*1 was observed throughout the development stage. Most significantly, no expression of *CycB1;*1 was detected throughout both the induction and development stages when 2,4-D was absent from the induction medium (B_5IV^{-D}). In general, the expression pattern of *CycA2* was similar to that of *cdc2a* during the induction and development stages, but the expression appeared slightly later and the level of the expression was generally weak compared to *cdc2a* (Figure 3.1, 3.5, table 3.2, 3.3).

Table 3.3: Expression of cell cycle genes in tobacco detected by histochemical GUS assay

Day	*cdc2a* expression		*CycB1;*1 expression		*CycA2* expression	
Induction stage	B_5IV	B_5IV^{-D}	B_5IV	B_5IV^{-D}	B_5IV	B_5IV^{-D}
1	−	−	−	−	−	−
3	+	+	+++	−	−	−
5	+	+	++++	−	+	−
7	++	++	++++	±	+	+
10	++++	+++	+++	−	+	++
14	++	+	−	−	++	++
18	++	+	−	−	++	+++
20	+++	+	++	−	++	+++
Development stage	B_53M	B_53M	B_53M	B_53M	B_53M	B_53M
3	+++	+++	+	−	++	++
7	+++	++	−	−	−	+
11	+++	++	−	−	+	+
15	++++	++	−	−	−	−
21	++	++	−	−	−	−
28	++	+	±	−		
33	+	±	+	−		

Note: +, positive GUS staining; −, negative GUS staining; ±, GUS staining between negative and positive.

3.3.4 The effect of the duration of 2,4-D induction on *cdc2a* expression in alfalfa

In order to investigate the role of 2,4-D in the activation of cell division, the chopped leaf explants from transgenic plant *cdc2a* were treated with B_5IV medium (containing 2,4-D) for 2, 10 and 60 min, after which the treated materials were washed and cultured in B_5IV^{-D}

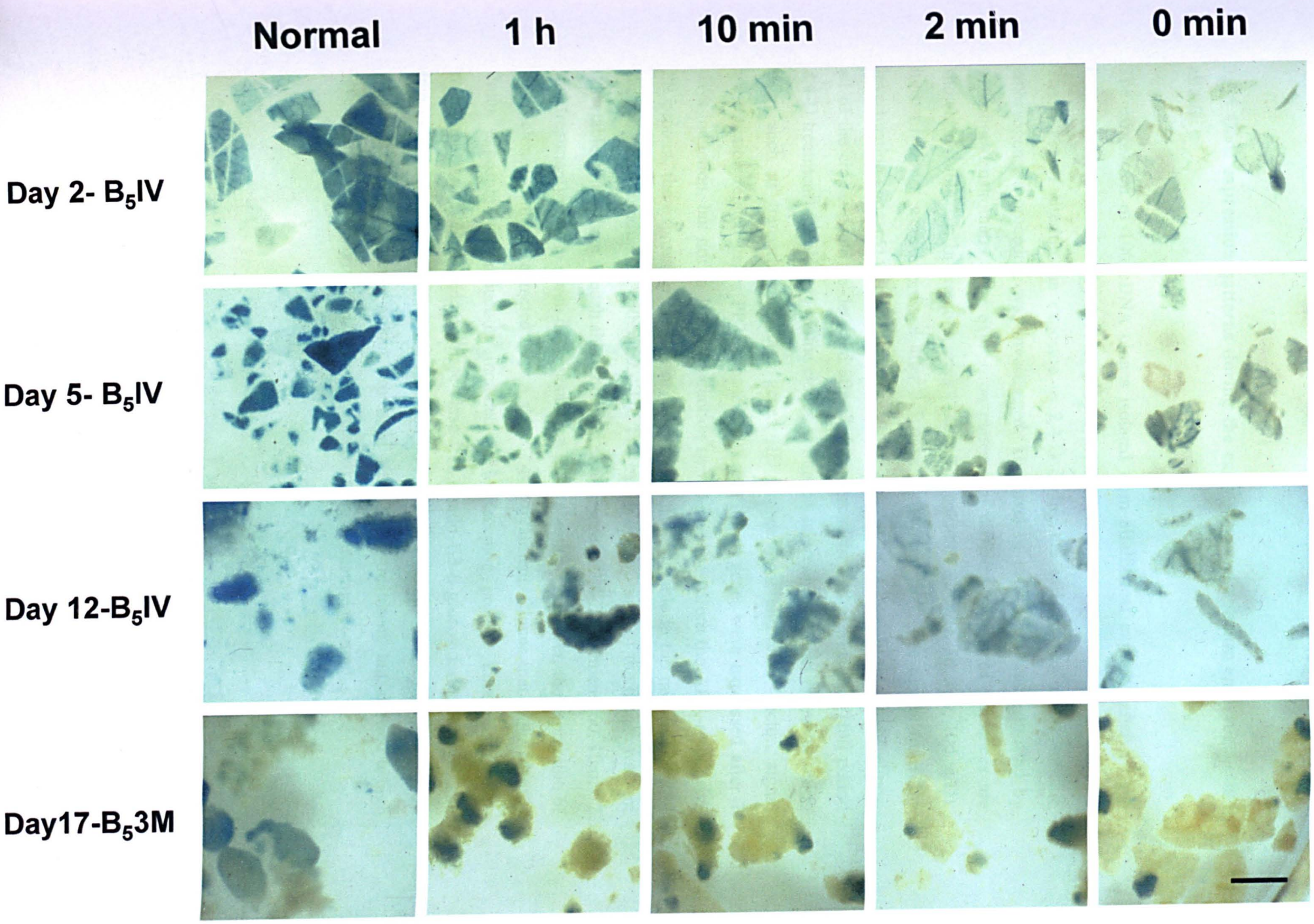
(B_5IV without 2,4-D) medium for 16 days. A swollen, spongy appearance of the leaf pieces was observed in every treatment within two weeks of induction, whereas the control tissue cultured in B_5IV^{-D} without any exposure to 2,4-D retained its original size and tissue organization. Histochemical GUS assays showed that the expression of *cdc2a* after short exposures to 2,4-D had the same pattern of expression as that found for the complete induction treatment (Figure 3.6). However, an increasing intensity of GUS staining was found with an increasing time of 2,4-D treatment (Figure 3.6 and table 3.4). On the other hand, no embryos were found in the development medium following 2, 10 and 60 min 2,4-D treatment. Expression of *cdc2a* in alfalfa with different time course treatment by 2,4-D detected by histochemical GUS assay was shown on table 3.4.

Table 3.4 Expression of *cdc2a* in alfalfa with different time course treatment by 2,4-D

Day	Medium	Expression of *cdc2a* detected by GUS assay				
Induction stage	B_5IV or B_5IV^{-D}	Normal	60 min	10 min	2 min	0 min
1		+	−	−	−	−
2		++	+	+	+	−
3		+++	+++	+++	++	+
5		++++	++++	+++	++	−
7		++++	++++	++++	+++	−
12		++++	++++	++	+	±
16		++++	++++	+	+	±
Development stage	$B_5$3M					
5		++++	+++	+	+	±
7		++++	+++	+	+	±
12		++++	++	+	+	−
17		+++	+	+	+	−
22		+++	+	+	+	−

Note: +, positive GUS staining; −, negative GUS staining; ±, GUS staining between negative and positive.

Figure 3.6 The influence of different durations of 2,4-D treatment during the induction of somatic embryogenesis in alfalfa and the expression of *cdc2a* revealed by GUS activity. Embryos were only observed from the positive control with full period 2,4-D induction treatment (bar= 700 μM).


Normal
1 h
10 min
2 min
0 min
Day 2- B5IV
Day 5- B5IV
Day 12-B5IV
Day17-B53M

3.3.5 Gene expression patterns during the early stages of somatic embryogenesis in alfalfa

The expression of 100 cDNA clones isolated from alfalfa leaf explants after 8 days induction, was examined by dot blot array hybridization.

The effect of altering the exposure to 2,4-D during the early stages of the somatic embryogenesis procedure was investigated. The level of expression was estimated by scanning densitometry. The expression patterns of different groups of sequences are shown in Table 3.1. In previous work (Table 3.1, columns 3d w/D, 5d w/D, 10d w/D), most of clones were expressed after 3-5 days 2,4-D induction. The results from this investigation appeared to be consistent with previous observations. In this study, almost all of the clones could be detected after 7 days normal induction treatment (full period 2,4-D treatment) (Table 3.1). Some clones of interest selected for further characterisation later, such as transcription factor genes HD Zipper gene and bZIP factor, signal transduction related genes PP2C Ppase and LysoPlipase gene, were expressed after 7 days induction, but not in 10 min induction treatment. Apart from the ABA-responsive PR protein, the expression of none of the clones was detectable in the negative control sample (7 days induction without 2,4-D). The expression of most of the clones was not detectable when given a much shorter period of exposure to 2,4-D treatment (60, 10 or 2 min treatment) prior to seven days incubation in its absence. However, the expression of some clones could be detected after a short 2,4-D treatment. Clones that required only 2min exposure for induction included EP1 Receptor protein kinase, phospho-mannomutase (PMM), endochitinase class I and endo-1,3-β-glucosidase , RSI-1b, and two unidentified proteins labelled GluRich and Y hyp. One clone, the receptor protein kinase-like PG inhibitor, was not expressed after 2 min treatment, but showed a high level of the expression in the 10 min, 60 min and normal treatment samples.

Table 3.1 Expression patterns of identified cDNA clones isolated by subtractive technique.

No	Identity of cloned sequences.	Lib 1	Lib 2	Lib 5	3d 0w	3d 0D	3d w/D	5d w/D	10d w/D	7d w/D	7d w/ 10m D	7d w/ 0D
	Transcription											
1-10	HD zipper	1			+/-	-	+/-	+	+/-	+	-	-
5-63	bZIP factor			1	+/-	+/-	+/-	++	+	++	-	-
1-28	IAA9	2			-	-	++	++	+	++++		
5-69	IAA 14			1	+++	++++	+++	++++	+++	+++	-	-
	Protein kinase											
5-77	Shaggy-like PK			1	-	-	-	-	-	++		
1-8	EP1 RecPK	1	2		-	-	+	+++	+++	+++		
1-9	PG inhibitor	1			+	+/-	++	++	++++	+++	+++	-
	Signal trans											
5-78	PP2C PPase			1	-	-	+	+	+	+		
2-131	LysoPlipase		1		-	-	-	-	+	+		
1-51	Calmodulin	1	2		++	+	+	+	++	++		
	Ribosomal proteins											
1-33	60S P2	1			-	-	+	++	+	+++		
1-49	60S L9	1			+	-	+	++	+	++		
1-6	60S L10/P0	2			++	++	++	++	++	+++		
1-43	60S L23	1			++	++	+++	++++	++	+++		
1-12	60S L44	1			++	+/-	+++	++++	++	+++		
5-97	60S L7A			1	++++	++++	+++	++++	++++	++		
5-81	60S L14		1	1	+	-	++	++	+	++		
5-66	60S L19			1	++	++	++	+++	+++	+++		
2-113	60S L3		1		++	++	++	+++	++	+++		
2-174	60S L30		2		++	++	++	+++	+	++		
2-169	60S L37A		3		+++	+++	+++	++++	+++	++		
1-11	60S L6P Mt	1			-	-	-	-	-	+++		
1-7	MtN25 L7/L12	1			++	++	++	+++	++	+++		
1-26	40S s7	1			++	-	+++	+++	+	+++		
1-47	40S s13	1			+	+/-	++	++	+	++		
1-44	40S s15	1			++	+/-	++	+++	+	++++		
1-24	40S s15A	1			++	+/-	++	++	+	+++		
2-163	40S s3		2		+	-	++	++	+	++		
2-104	40S s23		2		++++	++	+++	++++	++	++		
5-98	40S s3A cyc07		1	1	++	-	++	+++	+	++		
	Translation											
2-145	*Beet eEF2		1		+++	+++	+++	++++	++++	++++		

5-68	Translation eIF6			1		+	-	+	+	++		++		
	Ribogenesis												
5-99	Helicase-like			1		-	-	+/-	+	++		+++		
5-91	Helicase-like			1		-	-	-	+/-	+/-		+/-		
5-56	Nucleolin			1		+	-	+	++	++		++		
	Post-translation													
1-18	Ubiquitin	1				++	++	++	+++	+++		++		
2-160	Ubiquitin		1			-	-	+/-	+	-		+		
1-1	PMM	1				-	-	+	+	+/-		+++	+	
5-92	Pin1-like protein			1		-	-	+	+	+		++		
	Stress induced													
1-42	Endochitinase class I	1				-	-	++	++++	++		++++	++	
2-154	Endo-1,3-β-glucosidase		1			++	++	+	++	++++		++	++	
5-58	Endo-1,3-β-glucosidase			1		-	-	-	-	+		++		
5-65	Glucan 1,3-β-glucosidase			1		++	++	++	++	++		++		
2-133	PR protein		5			++	+/-	+	++++	++++		++		
1-20	ABA-responsive PR protein	1	1			+++	+++	+++	++++ +	+++		++++	++++	++++
1-32	WIP	1	3	1		++	-	++	++++	+++		++++		
	Redox													
2-125	Glutathione-S-transferase		1			++	++	+++	+++	+++		++		
2-148	Glutathione peroxidase		1			+/-	-	+/-	+	+		++		
5-85	SOD		1	1		++	++	++	++	++		++		
1-14	Ferredoxin	1				++	++	+++	++++	++		++		
5-73	Thioredoxin M			1		++	++	++	++	++		++		
2-175	Blue Cu protein	1	1			++	-	+	+	+		++		
5-94	Cyt B6-F comp			1		++	++	+	++	-		++		
5-82	Cyt c oxidase			1		+/-	+	+	++	++		++		
5-87	Cyt P450			1		+	-	+	++	++		++		
5-102	Oxidase		3	2		-	-	-	-	-		++		
1-4	ADH	1				+	-	++	+++	+++++		++++	++++	-
2-173	Hemoglobin II		10			++	-	++	+++	+++		+++		
	Metabolism													
1-16	Pyruvate kinase	1				-	-	-	-	-		-		
1-25	Fructokinase	1				+	-	+	++	++		+++		
	Transport													
1-21	MDR protein	1				-	-	-	-	-		-		
5-72	Aquaporin-like			1		+	-	+	+	++		++		
	Cell wall													
1-17	Extensin-like	1				+	-	++	+++	+		+++		

1-23	Proline-rich	1			-	-	+	+/-	-				
	Cytoskeletal												
1-54	Cortactin-like	1			+/-	+/-	+	++	+++		++		
2-151	Dynein		1		+/-	-	+	+	++		++		
5-76	Kinesin-like			1	+	-	+	+	+		+		
5-55	Actin			1	-	-	+	+	++		+++		
	Dev-reg proteins												
5-60	Mature somatic embryo DNA			1	-	-	-	+	+		++		
5-59	Early nodulin 93			1	-	-	+	+	++		++++		
2-138	Nod3		1		+/-	-	+	+	+		+++		
1-5	RSI-1	4	12?		+	-	+++	++++ +	+++		++		
5-75	RSI-1b			1	+	-	+	++	+++		+++	++	
5-86	DCL protein			1	-	-	-	-	-		+		
1-19	Unknown alfalfa protein	1			+	-	+	+	+		++		
5-74	Pollen-specific			5	+/-	-	+	+	+		++		
	Uncertain												
2-121	Germin-like		10		++	-	++	+++	++		+++		
2-124	DCRASH				++	-	+++	++++	+++		+++		
2-153	GluRich*		1		++	+	++	++++	+++		+++	++	
5-71	Penicillin-BP			1	++	++	++	++	++		++		
5-57	Histone H1			1	-	-	+/-	++	+		++		
2-176	Virus coat protein		1		++	+	+++	++++	++		++++		
5-100	Protamine			1	+	-	+	+	+		++		
1-3	Carrot DNA for transposon TdcI	1			++	++	++	++	+		+++		
5-101	A.thaliana BAC			1	-	-		+	+++		++		
2-178	A. thaliana BAC		1		++	+	+++	++++	+++		++++		
2-111	Y hyp		1		+/-	+/-	+	++	+++		++++	++	
5-79	Y hyp			1	-	-	-	-	-				
1-53	Hypothetical protein- yeast	1			-	-	+	+	+		++		
5-84	Yeast YDR09w			1	-	-	+	+	+		++		

Note:

(1). 3d 0W: the whole leaves cultured in induction medium B_5IV for 3 days;

(2). 3d 0D: the wounded leaf explants cultured in 2,4-D free induction medium B_5IV (B_5IV^{-D}) for 3 days;

(3). 3d W/D: the wounded leaf explants cultured in induction medium B_5IV for 3 days;

(4). 5d W/D: the wounded leaf explants cultured in induction medium B_5IV for 5 days;
(5). 10d W/D: the wounded leaf explants cultured in induction medium B_5IV for 10 days;
(6). 7d W/D: the wounded leaf explants cultured in induction medium B_5IV for 7 days;
(7). 7d W/10mD: the wounded leaf explants treated by 2,4-D for 10 min then cultured in 2,4-D free medium (B_5IV^{-D}) for 7 days;
(8). 7d W/0D: the wounded leaf explants cultured in 2,4-D free medium (B_5IV^{-D}) for 7 days.
(9). +, ++, +++, ++++, +++++ represent the level of the gene expression from low to high; –, represents negative expression of the gene.
Data (1)~(5) were obtained from previous work and the DNA samples used for dot blot are plasmid DNA (Shao's PhD thesis, 2000); data (6)~(8) were generated from this project and the DNA samples used for dot blot are PCR products.
(10) the results from 2 min 2,4-D treatment were not shown because the results were similar to 0 mins.
(11) Lib, the number of library.

3.3.6 Subcloning and sequencing of the RACE products derived from the subtractive clones

Several interesting clones (HD-Zip, bZIP, PP2C, LPL from subtractive clone) and cycMf2 were selected for further study because they are possibly involved in regulatory control mechanism during plant development. In this study, detailed information was obtained from subcloning and sequencing of RACE products derived from the original subtractive clones and they are shown in Table 3.5. The RACE reactions were generally successful, but that it required several attempts to obtain high quality sequence information from some of the RACE clones. Good quality sequence data were eventually obtained from the 3' RACE products of all four genes (bZip, PP2C, lysophospholipase and cyclin Mf2) (Figure 3.7, 3.8, 3.9, 3.10 and 3.11). However, the 5'RACE products proved more difficult to clone and sequence. As a result, there is no upstream sequence information for the bZip or cycMf2 genes. The 5' RACE sequence for the PP2C gene overlaps with the original cDNA clone but does not extend to the start of the mRNA sequence. The 5' RACE product of

lysophospholipase gene was not sequenced back to the primer sequence in the original cDNA clone. There is therefore a "floating" section of 5' sequence upstream of the cDNA clone.

Table 3.5 Subcloning and sequencing of RACE products

pGEM-T +Insert	Character	(SacI+SacII) Digestion		Samples Selected for Sequencing	Sequencing Number	Sequencing Data Obtained
		Band	Size			
pp 1'	5'-RACE of bZIP	2	a1			
pp 2'		2	a1	√	YZ19,28, 36, 60, 91	YZ60
pp 3'		2	a1	√	YZ1, 26, 29, 37, 61	
pp 4'		2	a1	√	YZ20, 30, 47, 58, 62	YZ62
pp 5'		2	b1	√	YZ2, 21,31, 48,59, 63, 87, 92	YZ92
qq 1	3'-RACE of bZIP	2	c2			
qq 2		2	c2	√	YZ3, 27	YZ27
qq 3		2	a2	√	YZ4	YZ4
qq 4		2	b2	√	YZ5, 22, 32, 38, 64	YZ5, 64
qq 5		2	a2	√	YZ6, 23, 33, 39, 65	YZ33, 65
rr 1	HD-Zip	2	a3	√	YZ7	
rr 2		1 (2 ?)	b3	√	YZ8	
rr 3		2	a3	√	YZ9	YZ9
rr 4		3	c3			
rr 5		2	a3	√	YZ10	
ss 1	5'-RACE of PP2C	2	c4	√	YZ11	
ss 2		1	a4			
ss 3		2	b4	√	YZ12	
ss 4		1	c4			
ss 5		1	a4			
tt 1	3'-RACE	1	b5			
tt 2		3	a5	√	YZ13	YZ13

tt 3	of PP2C	1	b5			
tt 4		0	-			
tt 5		2	c5	√	YZ14	YZ14
ww 1		2	c6	√	YZ15	
ww 2	5'-RACE	1	a6			
ww 3	of LPL	2	c6	√	YZ16, 93	
ww 4		2	b6	√	YZ17	YZ17
ww 5		1	a6			
xx 1		1	a7	√	YZ24, 34, 66, 88	
xx 2	3'-RACE	0	-			
xx 3	of LPL	0	-			
xx 4		2	b7	√	YZ18, 25, 35, 67, 89	
xx 5		1	a7			

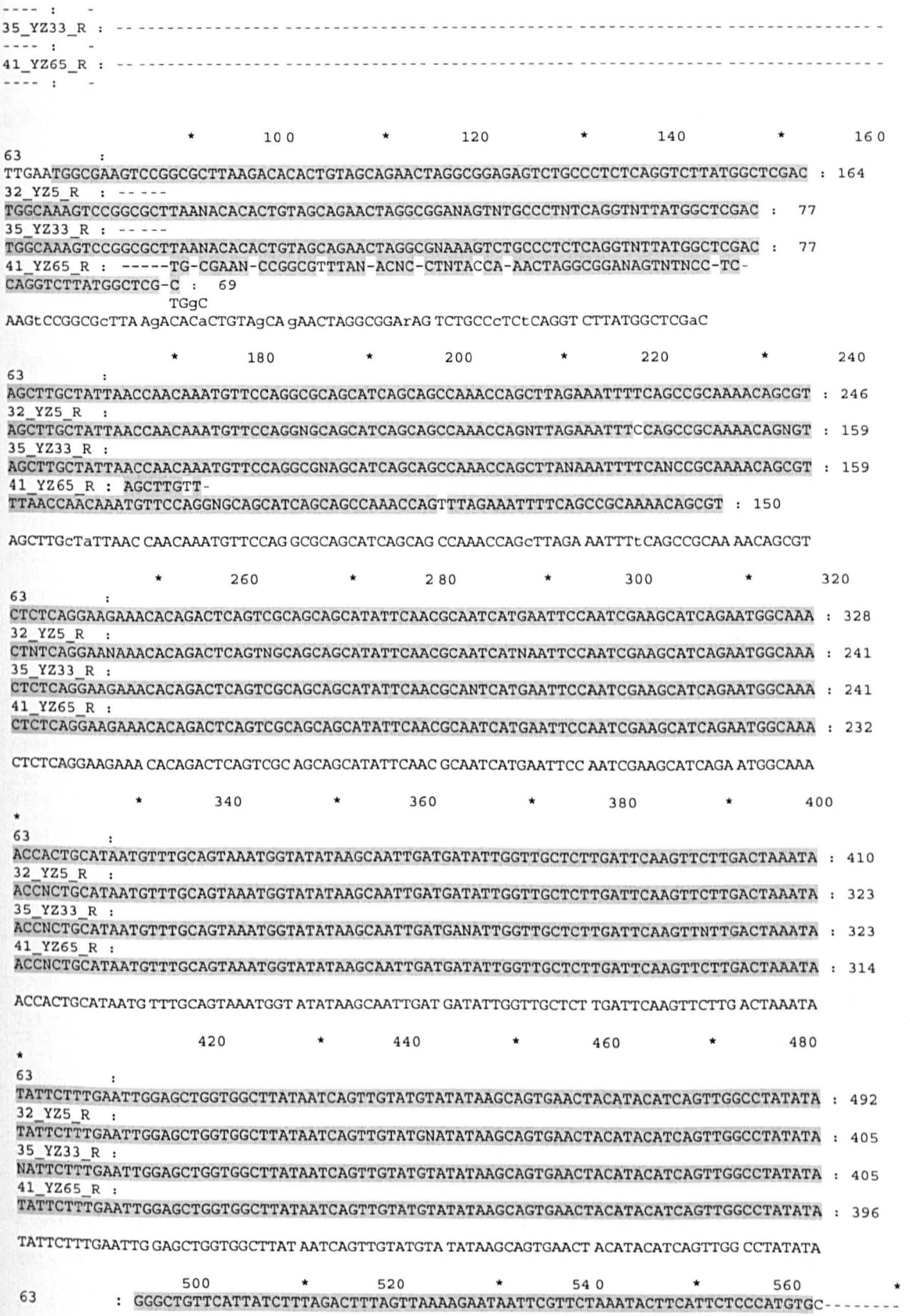
Note: a, b and c represent size of the bands from large to small.

The RACE alignments with the original cDNA clones (bZip, PP2C, LPL, cycMf2 and HD-Zip) are shown below. The further details of HD-Zip transcription factor gene will be discussed in Chapter 4.

The most primers for RACE were designed from overlapping sequence and the most original subtractive clone sequences were confirmed by overlapping sequence derived from RACE products, very small number bases did not agree between different sequences, suggesting the same clone with different termination.

Figure 3.7. bZIP RACE alignments with the original cDNA clones. The sequence in the first row number 63 was from the original subtractive clone. 32_YZ5_R, 35_YZ33_R, 41_YZ65_R are from subcloning of RACE products produced in this study. Yellow shade shows the agreement between different sequence samples. The grey shade shows the different bases between subclones.

```
                    *         20        *         40        *         60        *
80
63          :
CGGCGGCCGCCCGG GCAGGTACAAATTGAG GCTTCAATCCTTGGA ACAACAGTCCCAACT GAAAGATGCTCTGAA TGAGACT  :  82
32_YZ5_R    : ---------- ---------- ---------- ---------- ---------- ---------- ----------
```



```
---- : 562
32_YZ5_R  : GGGCTGTTCATTATCTTTAGACTTTAGTTAAAAGAATAATTCGTTCGAAAAAAAAAAAAAAAAAAAAAAAAA----
---- : 479
35_YZ33_R :
GGGCTGTTCATTATCTTTANACTTTAGTTAAAAGAATAATTCGTTNTAAATACTTCATTCTCCCATGTGTACTGGGAAGGAT : 487
41_YZ65_R :
GGGCTGTTCATTATCTTTAGACTTTAGTTAAAANAATAATTCGTTNTAAATACTTCATTNTCCCATGTGTACTGGGAAGGAT : 478
          GG GCTGTTCATTATCTT TAGACTTTAGTTAAA AGAATAATTCGTTCt AAAtActtcAttctc ccAtgtg a

              580         *       600         *
63        : ---------- ---------- ---------- ----- :   -
32_YZ5_R  : ---------- ---------- ---------- ------ :   -
35_YZ33_R : TTTTTCATGCTAAAAAAAAAAAAAAAAAAAAAAAAAAAA : 525
41_YZ65_R : TTTTTCATGCTAAAAAAAAAAAAAAAAAAAAAAAAAAAA : 516
```

Figure 3.8 PP2C RACE alignments with the original cDNA clones:

```
                *        20         *        40         *        60         *        80
78MOD : ---------- ---------- ---------- ---------- ---------- ---------- ----------
---- :   -
cym23 :
TNNTTTGATGCNTG GCCNACGCCCNATGC NNCCGGCCGCCNTGG CNGNCGCNGGAATTC NACTCTGANACGACT GANTNTAGGNNA :
86
YZ13  : ---------- ---------- ---------- ---------- ---------- ---------- ----------
---- :   -
YZ14  : ---------- ---------- ---------- ---------- ---------- ---------- ----------
---- :   -

          *       100         *       120         *       140         *       160
*
78MOD : ---------- ---------- ---------- ---------- ---------- ---------- ----------
---- :   -
cym23 :
NNGNNTNATCACCT NCAAGTCAGGTTCAT GCTGCTGATGATACA CCTGTTAGTGGTGGA GGGCTCAGCCAGAAT GGAAAATTCAGC :
172
YZ13  : ---------- ---------- ---------- ---------- ---------- ---------- ----------
---- :   -
YZ14  : ---------- ---------- ---------- ---------- ---------- ---------- ----------
---- :   -

             180         *       200         *       220         *       240         *
2
78MOD : ---------- ---------- ---------- ---------- ---------- ---------- ----------
---- :   -
cym23 :
TATGGATATGCTAGC TCCCCTGGCAAGAGA TCTTCAATGGAAGAT TTTTATGAGACAAGA ATTGACGGCGTTGAT GGCGAAGTTGT :
258
YZ13  : ---------- ---------- ---------- ---------- ---------- ---------- ----------
---- :   -
YZ14  : ---------- ---------- ---------- ---------- ---------- ---------- ----------
---- :   -

        60         *       280         *       300         *       320         *       340
78MOD : ---------- ---------- ---------- ---------- ---------- ---------- ----------
---- :   -
cym23 :
TGGCCTTTTTGGAG TTTTTGATGGTCATG GTGGTGCTCGCGCTG CTGAGTATGTCAAGC AAAACCTATTTAGTA ATTTGATCAGCC :
344
YZ13  : ---------- ---------- ---------- ---------- ---------- ---------- ----------
---- :   -
YZ14  : ---------- ---------- ---------- ---------- ---------- ---------- ----------
```

```
---- :   -

            *        360          *        380          *        400          *        420
*
78MOD : ------ ---------- ---------- ---------- ---------- ---------- ---------- --
CTGAAATCAGAGAATAAT :  18
cym23 :
ACCCGAAATTCATTTCTGACACCAAATCTGCCATAGCTGATGCGTATACGCATACCGACTCTGAATTTCTGAAATCAGAGAATAAT :
430
YZ13  : ------ ---------- ---------- ---------- ---------- ---------- ---------- ----------
---- :   -
YZ14  : ------ ---------- ---------- ---------- ---------- ---------- ---------- ----------
---- :   -

               440          *        460          *        480          *        500          *
78MOD :
CAAAACAGAGATGCTGGATCAACTGCTTCCACTGCCATTCTTGTTGGTGACCGTTTGCTTGTTGCAAATGTTGGGGACTCCAGAGC :
104
cym23 :
CAAAACAGAGATGCTGGATCAACTGCTTCCACTGCCATTCTTGTTGGTGACCGTTTGCTTGTTGCAAATGTTGGGGACTCCAGAGC :
516
YZ13  : ------ ---------- ---------- ---------- ---------- ---------- ---------- ----------
---- :   -
YZ14  : ------ ---------- ---------- ---------- ---------- ---------- ---------- ----------
---- :   -

        520          *        540          *        560          *        580          *
600
78MOD :
TGTTATATGCAGGGGTGGAAATGCCATTGCCGTTTCCCGAGATCACAAACCAGACCAAACCGATGAGAGGCAAAGGATAGAAGATG :
190
cym23 :
TGTTATATGCAGGGGTGGAAATGCCATTGCCGTTTNCCGAGATCACAAACCAGACCAAACCGATGAGAGGCANAGGATAGAAGATG :
602
YZ13  : ------ ---------- ---------- ---------- ---------- ---------- ---------- ----------
---- :   -
YZ14  : ------ ---------- ---------- ---------- ---------- ---------- ---------- ----------
---- :   -

               *        620          *        640          *        660          *        680
78MOD :
CGGGAGGCTTTGTTATGTGGGCTGGAACTTGGAGAGTCGGTGGTGTTCTTGCTGTTTCTCGCGCATTTGGTGATAGACTCCTTAAG :
276
cym23 : CGGGAGGCTTTGNTATGTGGGCTGGAACTTGGAGAGTCGGTGGTGT-CTTGCTGGTTCTCGCGCATTTG-
TGATAGACTCCTTAA-  : 685
YZ13  : ----------CGG-
GGTGTTCNTGNTGTTTCTCGCGCATTTGGTNATAGACTCCTTAAG :  48
YZ14  : ---------- -GGT-GGTGT-CTT-CTGTTTC-CGCGCATTTGG-
GATAGACTCCT-AAN :  43
                                    cGg GGTGT
CTTgCTGtTTCtCG CGCATTTGgtGATAG ACTCCTtAAg

        *        700          *        720          *        740          *        760          *
78MOD :
CAATACGTTGTTGCTGATCCGGAAATTCAGGAAGAAAAGGTCGATAGTTCTCTTGAGTTTCTTATACTGGCTAGTGACGGACTGTG :
362
cym23 : CAATATGTTGT-GCTGATCCG-AAAT-CAGGAAGAAAAG-TCGATAGT-CT-NTGAGTT-CTTAT-CTG-CTA-TGACG-
ACTGTG : 760
YZ13  :
CAATATGTTGTTGCTGATCCGNAAATCCAGGAAGAAAAGGNNNANAGTTCTCTTGAGTTTCTTANACTGGCTAGTGACGGACTGTG :
134
YZ14  : CAATACGTTGTTGCTGATCCG-
AAATTCAGAAAGAAAAGGTCGATAGTTCTCTTNAGTTTCTTATACTGGCTAGTGACGNACTGTG : 128
         CAATA  GTTGTtGCTGATCCG  AAAT
CAGgAAGAAAAGgT CGATAGTtCTcTTGA GTTtCTTATaCTGgC TAgTGACGgACTGTG

            780          *        800          *        820          *        840          *
```

```
860
78MOD :
GGATGTTGTCTCAAATGAGGAAGCTGTTGCTATGATTAAACCAATTGAGGATGCAGAAGAAGCAGCAAAGAGGCTGATGAAAGAAG :
448
cym23 : G-ATGT----------
---- : 765
YZ13  :
GNATGTTGTCTCAAATGAGGAAGCTGTTGCTATGATTAAACCAATTGAGGATGCAAAAGAAGCAGCAAAGAGGCTGATGAAAGAAG :
220
YZ14  :
GGATGTTGTCTCAAATGAGGAAGCTGTTGCTATGATTAAACCAATTGAGGATGCAGAAGAAGCAGCAAAGAGGCTGATGAAAGAAG :
214
        GgATGT tgtctcaaatgagga agctgttgctatgat taaaccaattgagga tgca
aagaagcagcaaag aggctgatgaaagaa g

                  *        880          *        900          *        920          *        940
78MOD :
CGTATCAGAGAGGTAGTTCTGACAACATTACTTGTGTCGTCATTCGTTTCCTGATGAACAATCAAGGTTCTTCGTCTCGTAATAGC :
534
cym23 : ----------
---- :   -
YZ13  :
NGNATCANAGAGGTAGTTCTGACAACATTACTTGNGTCGTCGTTCGTTTCCTNATGAACAATCAAGGTTNTTCGTCTCGTAATAGC :
306
YZ14  :
CGTATCAGAGAGGTAGTTCTGACAACATTACTTGTGTCGTCGTTCGTTTCCTNATGAACAATCAAGGTTCTTCGTCTCGTAATAGC :
300
        cgtatc agagaggtagttctg acaacattacttgtg tcgtc
ttcgtttcctgatg aacaatcaaggttct tcgtctcgtaatagc

          *        960          *        980          *       1 000          *       1020
*
78MOD : TCTGGCTAAGTGTACT----------
---- : 550
cym23 : ----------
---- :   -
YZ13  :
TCTGGCTAAGTGTACTTCAAAACATANAATTGTGTCAAAATCAGAGTTAATTATAGNAGGACATTGTTCCTTGTAACTGTGTGTAC :
392
YZ14  :
TCTGGCTAAGTGTACTTCAAAACATANAATTGTGTCAAAATCAGAGTTAATTATAGGAGGACATTGTTCCTTGTAACTGTGTGTAC :
386
        tctggc taagtgtact

            10 40          *       1060          *       1080          *       11 00          *
11
78MOD : ----------
---- :   -
cym23 : ----------
---- :   -
YZ13  : TACCATGCAAGCATAGGAATCAAAAGTTAANCTTATTATGGTCCTCTAGCATAGATATGTTGCTANCAAAAAAAAAAAAA---
---- : 471
YZ14  :
TNCCATGCAAGCATAGGAATCAAAAGTTAAACTTATTATGGTCCTCTAGCATAGATATGTTGCTATCACTCTCTTACTTCCATATT :
472


        20          *       114 0          *       1160          *       1180          *       120 0
78MOD : ----------
---- :   -
cym23 : ----------
---- :   -
YZ13  : ----------
---- :   -
YZ14  :
TTGGGATCCTATTT GTATTGTAGCTAGGA AAAATCGTTTTGTAA ANAGTGTGGCCAGAG TTTTTAACTTCCCAT ACAAATGTCTGC :
558


          *       1220          *       1240          *
78MOD : ---------- :   -
```

```
cym23 : ------ ---------- ---------- ---------- -- :  -
YZ13  : ------ ---------- ---------- ---------- -- :  -
YZ14  : ATGTAA ATGTGGTGGTGGTGT TCTCATTAATTCTCT TCCTTCCAAAAAAAA AA : 611
```

Figure 3.9 LPL RACE alignments with the original cDNA clones:

```
                    *         20          *         40          *         60          *
80          *
131mod    : --- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -
---------- :  -
29g32-2   : --- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -
---------- :  -
YZ17edit  :
CGCGGGATTGATCA CTTTAACTCGTGTGA GAGTCACGAGAGTAA CAAAGAAAAAGAGAG GTGAATCACAAGAGT CACAATGGAGAGTGG T
TCAG  :  94
MF16      : --- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -
---------- :  -

              100         *         120         *         140         *         160         *
180
131mod    : --- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -
---------- :  -
29g32-2   : --- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -
---------- :  -
YZ17edit  :
GTAAGAGAAAAAGT GGTGGTGGTGGTGGA ATACTTCGATTTTCT ATGGTGTTAGAAATA ATGCTTTGTTTTGTC GCTCTTGGTTCCCGG T
TTAC  : 188
MF16      : --- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -
---------- :  -

            *         200         *         220         *         240         *         260
*         280
131mod    : --- ---------- ---------- ---------- ---------- ---------- ---------- ----------
GAGATTCCTGGGAtAC  :  16
29g32-2   : --- ---------- ---------- ---------- ---------- ---------- ---------- ----------
GAGAATCCTGGGATAC  :  16
YZ17edit  : GGT GATCTTAAGGGTCTT ATGATCATTATTCAC GGGCCTTAATGTCCA CAGCCGGTAAGATAT TGCAGAT---------
---------- : 258
MF16      : --- ---------- ---------- ---------- ---------- ---------- ---------- ----------
---------- :  -

                    *         300         *         320         *         340         *         360
*
131mod    :
CATGCTTTCTCTTTGGTCACTCAACTGGAGGGGCTGTGGTTTTGAAAGCGGCATCATGCCCTCACATTGAAGTGATGGTAGAAGGATTCA
TTTT  : 110
29g32-2   :
CATGCTTTCTCTTTGGTCACTCAACTGGAGGGGCTGTGGTTTTGAAAGCGGCATCATGCCCTCACATTGAAGTGATGGTAGAAGGAATCA
TTTT  : 110
YZ17edit  : --- ---------- ---------- ---------- ---------- ---------- ---------- ----------
---------- :  -
MF16      : ----------CAA-TG-AGGG-CT-TG-TTT--AAA-CG-C--C--CCCCCCCCATN-AAGT-ATG-
TN-AAG-AT-C-TTTT  :  53
                          caa tg aggg ct tg ttt  aaa cg c  c   ccc c catt aagt atg
ta aag a  c tttt

            38 0        *         400         *         420         *         44 0        *
460         *
131mod    :
AACATCACCAGCTTTGCGTGTGAAGCCATCTCATCCAATAGTTGGcGCTGTTGCTCCAATATTTTCTCTGGTAGCTCCAAGGTTCCAGTT
CAAA  : 204
29g32-2   :
```

```
AACATCACCAGCTTTGCGTGTGAAGCCATCTCATCCAATAGTTGGCGCTGTTCGTCCAATATTTTCTCTGGTACGTCCAAGGTTCCAGTT
CAAA : 204
YZ17edit : ---------- : -
MF16     : A-CAN--NCAGCTT--GGTG-GAA--CNTCTC-TCCAN-AGT-GGCN-TGT-GC-CCAAN-TTTT-TNTGG-AGC-
CCCAGGTTCCATTT--AA : 127
           a cat  ccagctt   g tg gaa  catctc  tccaa agt ggcg  tgt    ccaat tt tt tctgg a   cc
aggttcca tt  a a

                  480         *       500         *       5 20         *       540
*       560
131mod   :
GGAGCAAACAAAAGAGGCATTCCAGTTTCTAGGGTCCCAGCAGCTTTGTTGGCAAAGTACTCTGATCCTCTGGTCTACACAGGACCTATT
AGAG : 298
29g32-2  : GGAGCAAACAAAAGAGGCATT---------- : 225
YZ17edit : ---------- : -
MF16     : G-AGCA--CAAAAAAG-CAT-CCAGTTT-TAGGNTCC-A-CAGCTTTGTTG-CAAAG-ACTCTGATCCTNTG-TCT-
CCCAGNACCTATTAGAG : 209
           g agca  caaaa ag cat

                *       580         *       600         *       620         *       640
*       6
131mod   :
TTCGAACTGGCCATGAAATACTGCGAATATCATCATACTTAATGCGAAACTTCAAGTCTGTGACAGTACCATTCTTTGTCCTGCATGGAA
CTGC : 392
29g32-2  : ---------- : -
YZ17edit : ---------- : -
MF16     : TTCGAACTG-
CCATGAAATACTGCGAATATCATCATACTTAATGCGAAACTTCAAGTCTGTGCCAGTACCATTCTTTGTCCTGCATGGAACTGC : 302

          60         *       680         *       700         *       720         *
740        *
131mod   :
TGATAAAGTAACTGATCCATTGGCTTCACAAGATTTATACAATAAGGCAGCTTCTGAGTTCAAAGACATAAAGCTTTATGATGGTTTCTT
GCAC : 486
29g32-2  : ---------- : -
YZ17edit : ---------- : -
MF16     :
TGATAAAGTAACTGATCCATTGGCTTCACAAGATTTATACAATAAGGCAGCTTCTGAGTTCAAAGACATAAAGCTTTATGATGGTTTNTT
GCAC : 396

                760         *       780         *       800         *       820         *
840
131mod   :
GACCTTTTGTTTGAGCCGGAGCGTGAAGAGATAGCTCAGGACATTATCAACTGGATGGAAAACAGATTATTTACTAGCATTTGAAAATGT
CAAT : 580
29g32-2  : ---------- : -
YZ17edit : ---------- : -
MF16     :
GACCTTTTGTTTGAGCCGGAGCGTGAANAGATAGCTCAGGACATTATCAACTGGATGGAAAACAGATTATTTACTAGCATTTGAAAATGT
CAAT : 490

              *       860         *       88 0         *       900         *       920
*       940
131mod   :
AATGGAAGGTTGATAGAGAAGTCAGCACCAAAAAAAAGGGGGGGTTGCCAACTTTCTAACTACCAAAGTTGGCAAGTGTGTAGAAAGTGA
AGTC : 674
29g32-2  : ---------- : -
YZ17edit : ----------
```

```
---------- :   -
MF16      :
AATGGAAGGTTGATANANAAGTCAGCNCCAAAAAAAAGGGGGGGTTGCCAACTTTCTAACTACCAAAGTTGGCAAGTGTGTANAAAGTGA
AGTC : 584


                    *        960          *        980          *       1000          *
1020         *
131mod    :
GCCCCGAAGAGTGGGTGGCGGAAGCAGATAGGCTATTCATATTTTATTTTATTTAGGTGTTGGACATATTTATTAATTAAATTATATATT
GTGT : 768
29g32-2   : ---------- ---------- ---------- ---------- ---------- ---------- ---------- -
---------- :   -
YZ17edit  : ---------- ---------- ---------- ---------- ---------- ---------- ---------- -
---------- :   -
MF16      :
GCCCCNAANAGTGGGTGGCGGAAGCANATAGGTTATTCATATTTTATTTTATTTAGGTGTTGGACATATTTATTAATTAAATTATATATT
GTGT : 678


            1040         *       1060         *       1080         *       1100         *
1120
131mod    : GTAGGGATTGT-----CGTCCATTGAGAAATTTGTA---------- ---------- ---------- -
---------- : 799
29g32-2   : ---------- ---------- ---------- ---------- ---------- ---------- ---------- -
---------- :   -
YZ17edit  : ---------- ---------- ---------- ---------- ---------- ---------- ---------- -
---------- :   -
MF16      :
GTAGGGATTGTATTGGCGTCCATTNANAAATTTGTACATTCAGCTGGCTTAAATTTCAG TGATTTTCCGTGGCC CTAAATCCTGATAAT A
AATC : 772


          *       1140
131mod    : ---------- :   -
29g32-2   : ---------- :   -
YZ17edit  : ---------- :   -
MF16      : CCC CAAGAAAAAAAAA : 788
```

Figure 3.10. cycMf2 RACE alignments with the original cDNA clones:

```
                    *         20          *         40          *         60          *
80
cycmf2    :
CGAAGAGATGTGTGCTCCTCGAGTGGAAGAATTTTGCTTCATCACAGATAATACATACACAAAAGAAGAGGTAGTAAAAATG :  82
68_YZ70_R : ---------- ---------- ---------- ---------- ---------- ---------- ---------- -
---- :   -
YZ94_u    :
CGAAGAGATGTGTGCTCCTCGAGTGGAAGAATTTTGCTTCATCACAGATAATACATACACAAAAGAAGAGGTAGTAAAAATG :  82

cgaagagatgtgtg ctcctcgagtggaag aattttgcttcatca cagataatacataca caaaagaagaggtag taaaaatg


                    *        100          *        120          *        140          *        160
cycmf2    :
GAGGAGGAAGTTTTAAACCTTCTGCGTTTTCAGTTATCTGTTCCCACAACCAAAACATTTCTCAGGAGATTCATCCAAGCAG : 164
68_YZ70_R : ---------- ---------- ---------- ---------- ---------- ---------- ---------- -
-CA- :   2
YZ94_u    :
GAGAAGGAAGTTTTAAACCTTCTGCGTTTTCAGTTATCTGTTCCCACAACCAAAACATTTCTCAGGAGATTCATCCAAGCAG : 164
          ga g
aggaagttttaaac cttctgcgttttcag ttatctgttcccaca accaaaacatttctc aggagattcatccaa gCAg


                  *        180          *        200          *        220          *        240
cycmf2    : CACAATCTTCTTACAAGGTTCCTCTTGCTGAACTGGAATTCCTGGCTAAG---------- ---------- -
---- : 214
68_YZ70_R : CACCATN-TCT-ACAAG--TCCTC-TG-TG-ACTG--ATTC-TG-CA-AT-ATT-A-CANAGC-CACTCT-G-TGAA-
ACAG :  65
YZ94_u    :
```

```
CACAATCTTCTTACAAGGTTCCTCTTGCTGAACTGGAATTCCTGGCAAATTATTTAGCAGAGCTCACTCTTGTTGAATACAG : 246
          CA CaATCtTCTtACAAG gtTCCTCtTGcTGaA CTGgaATTCcTGgCa aAt att a cagag c cactct g tgaa
acag

                *        260          *        2 80          *        300          *        320
cycmf2    : ----------------------------------------------------------------------------------
---- :   -
68_YZ70_R : -TTCT-ACAGTT-CTNC-TTCTCG-GTG-CTGCT-CTGCTG-AT-CCTT-CCAGATG-ACCC-CACCCAC-
CAGAACATCCA : 134
YZ94_u    :
CTTCTTACAGTTTCTACCTTCTCGTGTGGCTGCTTCTGCTGTATTCCTTGCCAGATGGACCCTCAACCACTCAGAACATCCA : 328
            t tct acagtt ctac ttctcg gtg ctg ct ctgctg at cc tt ccagatg accc ca ccac
cagaacatcca

            *        340          *        360          *        380          *        400
*
cycmf2    : ----------------------------------------------------------------------------------
---- :   -
68_YZ70_R : TGGACTACAACTCTGGAGCATT-TACCAACTACAAAGCT-
CAGAGCTAAAACCAGTTGTTCTTGCACTGGAAGATCTGCAAC : 214
YZ94_u    :
TGGACTACAACTCTGGAGCATTTTACCAACTACAAAGCTTCAGAGCTAAAACCAGTTGTTCTTGCACTGGAAGATCTGCAAC : 410
            tg gactacaactctgga gcatt taccaacta caaagct
cagagctaaaacca gttgttcttgcactg gaagatctgcaac

                    420          *        440          *        460          *        480
*
cycmf2    : ----------------------------------------------------------------------------------
---- :   -
68_YZ70_R :
TTAATACCAAAGGTTGTTCTCTCCATGCTATACGTGAGAAATATAAACATGAGAAGTTCAATGGTGTGGCAAAACTGTCTCC : 296
YZ94_u    :
TTAATACCAAAGGTTGTTCTCTCCATGCTATACGTGAGAAATATAAACATGAGAAGTTCAATGGTGTGGCAAAACTGTCTCC : 492

ttaataccaaaggt tgttctctccatgct atacgtgagaaatat aaacatgagaagttc aatggtgtggcaaaa ctgtctcc

                  500          *        520          *        54 0          *        560          *
cycmf2    : ----------------------------------------------------------------------------------
---- :   -
68_YZ70_R :
AAAACCGGTGCAGTCACTGTTCCAGGCTCAAGTGTAAATTTATGCATTTNTAAGGACTCGATCATGACTCGGAATTTCCTGT : 378
YZ94_u    :
AAAACCGGTGCAGTCACTGTTCCAGGCTCAAGTGTAAATTTATGCATTTCTAAGGACTCGATCATGACTCGGAATTTCCTGT : 574

aaaaccggtgcagt cactgttccaggctc aagtgtaaatttatg catttctaaggactc gatcatgactcggaa tttcctgt

                580          *        600          *        620          *        640          *
cycmf2    : ----------------------------------------------------------------------------------
---- :   -
68_YZ70_R :
ACAATNAGGGTGTTTTTATCATCAGTNGTCATATTGTTTACCCTTTACTCATTAGTTANGTAGTTGTAACATTC ATGTAGTT : 460
YZ94_u    : ACAATGAGGGTGTTTTTATCATCAGTCGTCATATTGTTTACCCTTTACTCATT----------------------------
---- : 627
            ac aatgagggtgttttt atcatcagtcgtcat attgtttacccttta ctcatt

                660          *        680          *        700          *        7 20          *
7
cycmf2    : ----------------------------------------------------------------------------------
---- :   -
68_YZ70_R :
GTAACATGTAANAT GATATCAATTTTTTG TTCTTNTAGATCTCC ATGTAACATGTTGAT ACAACTCAAACAGTG AGTTGAAG : 542
YZ94_u    : ----------------------------------------------------------------------------------
---- :   -


                40           *        760          *        780          *        800          *
cycmf2    : ----------------------------------------------------------------------------------
---- :   -
68_YZ70_R :
TACTGAGGTGAAGT AGAATACTTCATTTT GAGGTCGCCGTTATA ATTCAACAAAGCCAT GTAGCTGCTTTCAAA AAAAAAA : 623
YZ94_u    : ----------------------------------------------------------------------------------
```

--- : -

Figure 3.11 HD-Zip RACE alignments with the original cDNA clones:

```
                    *        20         *        40         *        60         *
80
HDZCOMB     :
GGCTCCCTCTCTCT CGCACGCACAC ACCAAAAAACCTCGCCGGGAAAACTCCATCTCCGGCCACCGGCGTTCTCCTTCCT :   80
34_YZ119_T : ----------------------------------------------------------------------
CCT :    3
90_YZ218_M : ----------------------------------------------------------------------
--- :    -
83_YZ211_M : ----------------------------------------------------------------------
CCT :    3
85_YZ213_M : ----------------------------------------------------------------------
--- :    -
66_YZ127_T : ----------------------------------------------------------------------
--- :    -
67_YZ127_T : ----------------------------------------------------------------------
--- :    -

                    *        100        *        120        *        140        *
160
HDZCOMB     :
CCGGTAAACCCTTCAGTTCAAAACTGAATCCCTTCAAAGACCACCGGAATCGGTGGTTTTTACCTTACAGTGCATGATGG :  160
34_YZ119_T :
CCGGTAAACCCTTCAGTTCAANACTGAATCCCTTCAAAGACCACCGGAATCGGNGGTTTTTACCTTACAGCGCATGATGG :   83
90_YZ218_M : ----------------------------------------------------------------------
--- :    -
83_YZ211_M :
CCGGTAAACCCTTCAGTTCAAAACTGAATCCCTTCAAAGACCACCGGAATCGGTGGTTTTTACCTTACAGTGCATGATGG :   83
85_YZ213_M : ----------------------------------------------------------------------
--- :    -
66_YZ127_T : ----------------------------------------------------------------------
--- :    -
67_YZ127_T : ----------------------------------------------------------------------
--- :    -

                    *        180        *        200        *        220        *
240
HDZCOMB     :
GATCTTGCATATGTCCGTTAGAAACTCCAGCAAGGTTGCTTTGGACAACAAGTTTCTTCCGTCATAAGTTAATGATCTTT :  240
34_YZ119_T :
GATCTTGCATATGTCCGTTAGAAACTCCAGCAAGGTTGCTTTGGACAACAAGTTTCTTCCGTCATAAGTTAATGATCTTT :  163
90_YZ218_M : ----------------------------------------------------------------------
--- :    -
83_YZ211_M :
GATCTTGCATATGTCCGTTAGAAACTCCAGCAAGGTTGCTTTGGACAACAAGTTTCTTCCGTCATAAGTTAATGATCTTT :  163
85_YZ213_M : ----------------------------------------------------------------------
--- :    -
66_YZ127_T : ----------------------------------------------------------------------
--- :    -
67_YZ127_T : ----------------------------------------------------------------------
--- :    -

                    *        260        *        280        *        300        *
320
HDZCOMB     :
TAATCCACAAATCATCATAGAAATATTTCTATAAAAAAAATATCAAGTTTGGAATTAGAAAGAAAAAATATATATATCAT :  320
34_YZ119_T :
TAATCCACAAACCATCATAGAAATATTTCTATAAAAAAAATATCAAGTTTGGAATTAGAAAGAAAAAATATATATATCAT :  243
90_YZ218_M : ----------------------------------------------------------------------
--- :    -
83_YZ211_M :
```

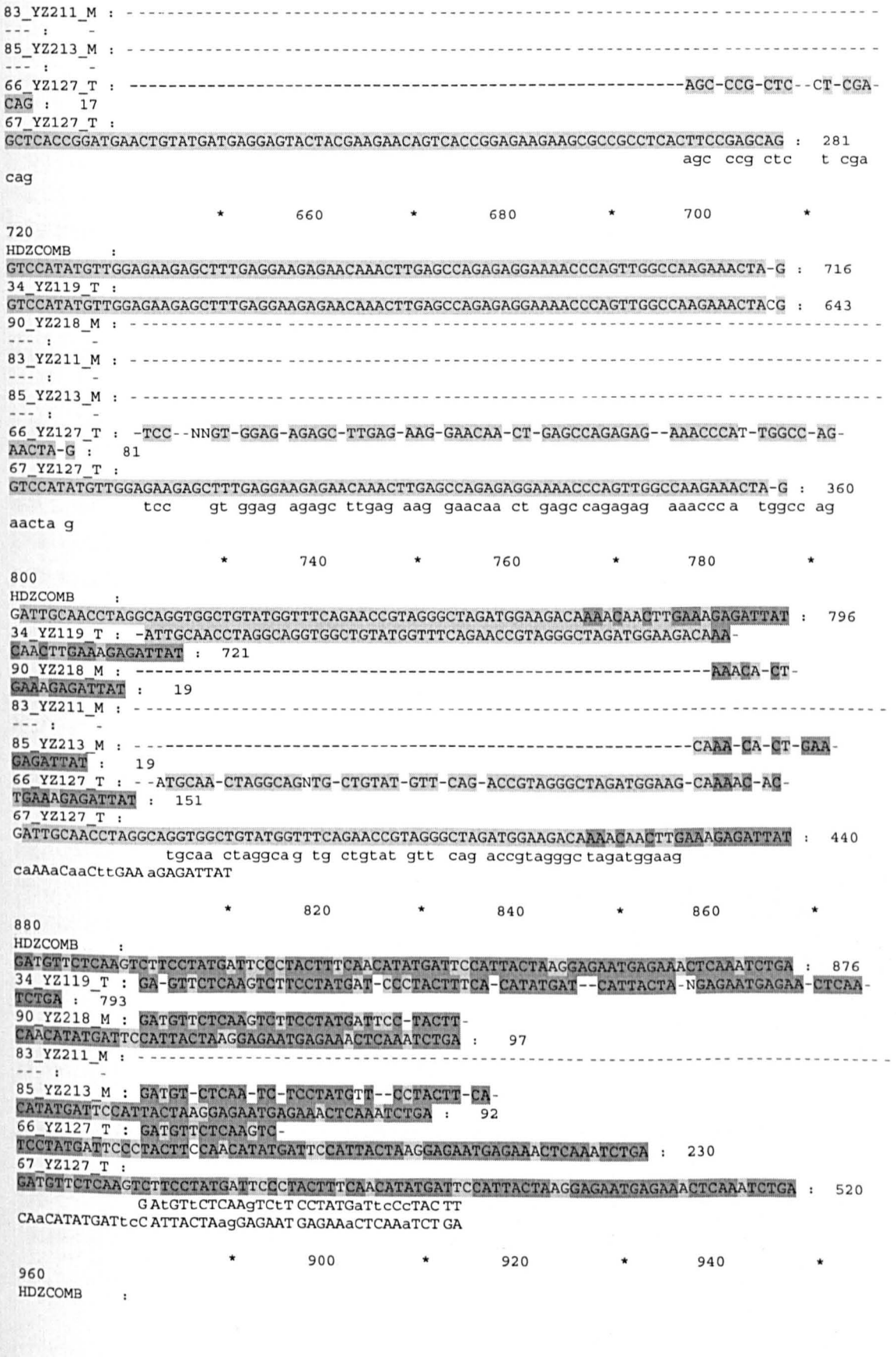
```
TAATCCACAAACCATCATAGGAATATTTCTATAAAAAAAAATATCAAGTTTGGAATTAGAAAGAAAAAATATATATATCAT :  243
85_YZ213_M : ----------------------------------------------------------------------------------------
--- :    -
66_YZ127_T : ----------------------------------------------------------------------------------------
--- :    -
67_YZ127_T : ----------------------------------------------------------------------------------------
--- :    -


                    *       340         *       360         *       380         *
400
HDZCOMB    :
TTTTTGTTTCTGATTCTGAAAGATTAATAGTATTAAGAGAAAATTTGTAGCACAACCTTTAACGTGAAGTTTTGAAGTTT :  400
34_YZ119_T :
TTTTTGTTTCTGATTCTGAAAGATTAATAGTATTAAGAGAAAATTTGTAGCACAACCTTTAACGTGAAGTTTTGAAGTTT :  323
90_YZ218_M : ----------------------------------------------------------------------------------------
--- :    -
83_YZ211_M : TTTTTGTTTCTGATTCTGAAAGATTAATAGTATTAAGAGAAAATTTGTAGCACAACCTTTAACGTGAAG--------
--- :  312
85_YZ213_M : ----------------------------------------------------------------------------------------
--- :    -
66_YZ127_T : ----------------------------------------------------------------------------------------
--- :    -
67_YZ127_T : ------------------------------------GATCCT-
TGTANCACAACCTTTAACGTGAAGTNNTGAAGTTT :  41
                                                ga   t tgta cacaaccttta acgtgaag

                    *       420         *       440         *       460         *
480
HDZCOMB    :
GAAGTTGAAAAAATGGAGTCTGGTCGTCTTTACTTTGATTCTCCTGCTTGTCGTGGGAATAACATGAACATGCTGTTTCT :  480
34_YZ119_T :
GAAGTTGAAAAAATGGAGTCTGGTCGTCTTTACTTTGATTCTCCTGCTTGTCGTGGGAATAACATGAACATGCTGTTTCT :  403
90_YZ218_M : ----------------------------------------------------------------------------------------
--- :    -
83_YZ211_M : ----------------------------------------------------------------------------------------
--- :    -
85_YZ213_M : ----------------------------------------------------------------------------------------
--- :    -
66_YZ127_T : ----------------------------------------------------------------------------------------
--- :    -
67_YZ127_T :
GACCTTGAAAAAATGGAGTCTGGTCGTCTTTACTTTGATTCTCCTGCTTGTCGTGGGAATAACATGAACATGCTGTTTCT :  121


                    *       500         *       520         *       540         *
560
HDZCOMB    : TGGAAATGCTGATCTTGGTTTTCGAG---
GAAGGTCAATGATGAGCATGGGGGGAAGGCTCAAAAAGGAGACCTTTCTTTA :  557
34_YZ119_T :
TGGAAATGCTGATCTTGGTTTTCGAGCAGGAAGGTCAATGATGAGCATGGGGGGAAGGCTCAAAAAGGAGACCTTTCTTTA :  483
90_YZ218_M : ----------------------------------------------------------------------------------------
--- :    -
83_YZ211_M : ----------------------------------------------------------------------------------------
--- :    -
85_YZ213_M : ----------------------------------------------------------------------------------------
--- :    -
66_YZ127_T : ----------------------------------------------------------------------------------------
--- :    -
67_YZ127_T :
TGGAAATGCTGATCTTGGTTTTCGAGCAGGAAGGTCAATGATGAGCATGGGGGGAAGGCTCAAAAAGGAGACCTTTCTTTA :  201


                    *       580         *       600         *       620         *
640
HDZCOMB    :
GCTCACCGGATGAACTGTATGATGAGGAGTACTACGAAGAACAGTCACCGGAGAAGAAGCGCCGCCTCACTTCCGAGCAG :  637
34_YZ119_T :
GCTCACCGGATGAACTGTATGATGAGGAGTACTACGAAGAACAGTCACCGGAGAAGAAGCGCCGCCTCACTTCCGAGCAG :  563
90_YZ218_M : ----------------------------------------------------------------------------------------
--- :    -
```


83_YZ211_M :
85_YZ213_M :
66_YZ127_T :
CAG : 17
67_YZ127_T :
GCTCACCGGATGAACTGTATGATGAGGAGTACTACGAAGAACAGTCACCGGAGAAGAAGCGCCGCCTCACTTCCGAGCAG : 281
agc ccg ctc t cga
cag
660
680
700
720
HDZCOMB :
GTCCATATGTTGGAGAAGAGCTTTGAGGAAGAGAACAAACTTGAGCCAGAGAGGAAAACCCAGTTGGCCAAGAAACTA-G : 716
34_YZ119_T :
GTCCATATGTTGGAGAAGAGCTTTGAGGAAGAGAACAAACTTGAGCCAGAGAGGAAAACCCAGTTGGCCAAGAAACTACG : 643
90_YZ218_M :
83_YZ211_M :
85_YZ213_M :
66_YZ127_T : -TCC--NNGT-GGAG-AGAGC-TTGAG-AAG-GAACAA-CT-GAGCCAGAGAG--AAACCCAT-TGGCC-AG-
AACTA-G : 81
67_YZ127_T :
GTCCATATGTTGGAGAAGAGCTTTGAGGAAGAGAACAAACTTGAGCCAGAGAGGAAAACCCAGTTGGCCAAGAAACTA-G : 360
tcc gt ggag agagc ttgag aag gaacaa ct gagc cagagag aaaccc a tggcc ag
aacta g
740
760
780
800
HDZCOMB :
GATTGCAACCTAGGCAGGTGGCTGTATGGTTTCAGAACCGTAGGGCTAGATGGAAGACAAAACAACTTGAAAGAGATTAT : 796
34_YZ119_T : -ATTGCAACCTAGGCAGGTGGCTGTATGGTTTCAGAACCGTAGGGCTAGATGGAAGACAAA-
CAACTTGAAAGAGATTAT : 721
90_YZ218_M : AAACA-CT-
GAAAGAGATTAT : 19
83_YZ211_M :
85_YZ213_M : CAAA-CA-CT-GAA-
GAGATTAT : 19
66_YZ127_T : --ATGCAA-CTAGGCAGNTG-CTGTAT-GTT-CAG-ACCGTAGGGCTAGATGGAAG-CAAAAC-AC-
TGAAAGAGATTAT : 151
67_YZ127_T :
GATTGCAACCTAGGCAGGTGGCTGTATGGTTTCAGAACCGTAGGGCTAGATGGAAGACAAAACAACTTGAAAGAGATTAT : 440
tgcaa ctaggca g tg ctgtat gtt cag accgtagggc tagatggaag
caAAaCaaCttGAA aGAGATTAT
820
840
860
880
HDZCOMB :
GATGTTCTCAAGTCTTCCTATGATTCCCTACTTTCAACATATGATTCCATTACTAAGGAGAATGAGAAACTCAAATCTGA : 876
34_YZ119_T : GA-GTTCTCAAGTCTTCCTATGAT-CCCTACTTTCA-CATATGAT--CATTACTA-NGAGAATGAGAA-CTCAA-
TCTGA : 793
90_YZ218_M : GATGTTCTCAAGTCTTCCTATGATTCC-TACTT-
CAACATATGATTCCATTACTAAGGAGAATGAGAAACTCAAATCTGA : 97
83_YZ211_M :
85_YZ213_M : GATGT-CTCAA-TC-TCCTATGTT--CCTACTT-CA-
CATATGATTCCATTACTAAGGAGAATGAGAAACTCAAATCTGA : 92
66_YZ127_T : GATGTTCTCAAGTC-
TCCTATGATTCCCTACTTCCAACATATGATTCCATTACTAAGGAGAATGAGAAACTCAAATCTGA : 230
67_YZ127_T :
GATGTTCTCAAGTCTTCCTATGATTCCCTACTTTCAACATATGATTCCATTACTAAGGAGAATGAGAAACTCAAATCTGA : 520
G AtGTtCTCAAgTCtT CCTATGaTtcCcTAC TT
CAaCATATGATtcC ATTACTAagGAGAAT GAGAAaCTCAAaTCT GA
900
920
940
960
HDZCOMB :

```
GGTGGTATCCTTAAATGAGAAGCTTCAAGTTCAAGCTAAAGATATGCTTGAAGAACCTTTGTCAGAAAAGAAAGCTGATC :  956
34_YZ119_T : G-TGG-ATCCTTAAG-GAGA-GCT-CA-GT-CAA-CTAA-GAT-TGCTTGA-NAAC--TTG-CNGAAA-GAAG--
TG-NC :  855
90_YZ218_M :
GGTGGTATCCTTAAATGAGAAGCTTCAAGTTCAAGCTAAAGATATGCTTGAAGAACCTTTGTCAGAAAAGAAAGCTGATC :  177
83_YZ211_M : ----------
--- :    -
85_YZ213_M :
GGTGGTATCCTTAAATGAGAAGCTTCAAGTTCAAGCTAAAGATATGCTTGAAGAACCTTTGTCAGAAAAGAAAGCTGATC :  172
66_YZ127_T :
GGTGGTATCCTTAAATGAGAAGCTTCAAGTTCAAGCTAAAGATATGCTTGAAGAACCTTTGTCAGAAAAGAAAGCTGATC :  310
67_YZ127_T :
GGTGGTATCCTTAAATGAGAAGCTTCAAGTTCAAGCTAAAGATATGCTTGAAGAACCTTTGTCAGAAAAGAAAGCTGATC :  600

GgTGGtATCCTTAA atGAGAaGCTtCAaG TtCAAgCTAAaGATa TGCTTGAagAACctT TGtCaGAAAaGAAag cTGatC

                        *        980          *       1000          *       1020          *
1040
HDZCOMB     :
CACTTCCAGTGGACATAGCTCAGATTTTCAGCATCAGGGTGGATGACCACATGAGTTCTGGAAGCGTTGGAAGTGCAGTG : 1036
34_YZ119_T : C-CTTC-AGTGAATA---CTCA--ATTTC-NCA-CAGGG-G--NGNCC--ATGA----------
--- :  895
90_YZ218_M :
CACTTCCAGTGGACATAGCTCAGATTTTCAGCATCAGGGTGGATGACCACATGAGTTCTGGAAGCGTTGGAAGTGCAGTG :  257
83_YZ211_M : ----------
--- :    -
85_YZ213_M :
CACTTCCAGTGGACATAGCTCAGATTTTCAGCATCAGGGTGGATGACCACATGAGTTCTGGAAGCGTTGGAAGTGCAGTG :  252
66_YZ127_T :
CACTTCCAGTGGACATAGCTCAGATTTTCAGCATCAGGGTGGATGACCACATGAGTTCTGGAAGCGTTGGAAGTGCAGTG :  390
67_YZ127_T : CACTTCCAGTGGACATAGCTCAGATTTTCAGCATCAGGGTGGATGACCACATGAGTTCTGGAAGCG-
TGGAAGTGCAGTG :  679

CaCTTCcAGTGgAc AtagCTCAgatTTTC agCAtCAGGGtGgat GaCCacATGAgttct ggaagcgttggaagt gcagtg

                        *        1060         *       1080          *       1100          *
1120
HDZCOMB     :
GTGGACGAGAGTAGCCCCCGTGTCGTTGGTGTCATTGTTGACAGCGTCGATTCATACTTTCCAGCTGACAACTATGCTGG : 1116
34_YZ119_T : ----------
--- :    -
90_YZ218_M :
GTGGACGAGAGTAGCCCCCGTGTCGTTGGTGTCATTGTTGACAGCGTCGATTCATACTTTCCAGCTGACAACTATGCTGG :  337
83_YZ211_M : ----------
--- :    -
85_YZ213_M :
GTGGACGAGAGTAGCCCCCGTGTCGTTGGTGTCATTGTTGACAGCGTCGATTCGTACTTTCCAGCTGACAACTATGCTGG :  332
66_YZ127_T :
GTGGACGAGAGTAGCCCCCGTGTCGTTGGTGTCATTGTTGACAGCGTCGATTCATACTTTCCAGCTGACAACTATGCTGG :  470
67_YZ127_T : GTGGACGAGAGTA-NCCCCGTGTCG-TGGTGTCN-TGTTGAC-GCGTCGATTC-TACTTT-CAGCTGAC-
ACTATGCTGG :  752
             g tggacgagagtagcc cccgtgtcgttggtg tcattgttgacagcg tcgattc
tactttccagctga caactatgctgg

                        *        1140         *       1160          *       1180          *
1200
HDZCOMB     :
ATGTGTGGCTCCTATTGAAAGAGTTCAATCAGAGGAAGATGATGGGAGTGATGATGGGAGGAACTACTTTGATGTGTTTG : 1196
34_YZ119_T : ----------
--- :    -
90_YZ218_M :
ATGTGTGGCTCCTATTGAAAGAGTTCAATCAGAGGAAGATGATGGGAGTGATGATGGGAGGAACTACTTTGATGTGTTTG :  417
83_YZ211_M : ----------
--- :    -
85_YZ213_M :
ATGTGTGGCTCCTATTGAAAGAGTTCAATCAGAGGAAGATGATGGGAGTGATGATGGGAGGAACTACTTTGATGTGTTTG :  412
66_YZ127_T :
ATGTGTGGCTCCTATTGAAAGAGTTCAATCAGAGGAAGATGATGGGAGTGATGATGGGAGGAACTACTTTGATGTGTTTG :  550
67_YZ127_T : N-G-GTGGCT-CTA-TG-AAGAGT----------
--- :  771
```

```
atgtgtggctccta ttgaaagagttcaat cagaggaagatgatg ggagtgatgatggga ggaactactttgatg tgtttg

                          *       1220          *       1240          *       1260          *
1280
HDZCOMB     :
TTGCATCTGAAACTGAGCAACATAACCATGAAGAGGGAGAGGCACTGAATTGGTGGGGTAATATGTATTATGTTGCATAA : 1276
34_YZ119_T : ----------  ----------  ----------  ----------  ----------  ----------
---  :     -
90_YZ218_M :
TTGCATCTGAAACTGAGCAACATAACCATGAAGAGGGAGAGGCACTGAATTGGTGGGGTAATATGTATTATGTTGCATAA :  497
83_YZ211_M : ----------  ----------  ----------  ----------  ----------  ----------
---  :     -
85_YZ213_M :
TTGCATCTGAAACTGAGCAACATAACCATGAAGAGGGAGAGGCACTGAATTGGTGGGGTAATATGTATTATGTTGCATAA :  492
66_YZ127_T :
TTGCATCTGAAACTGAGCAACATAACCATGAAGAGGGAGAGGCACTGAATTGGTGGGGTAATATGTATTATGTTGCATAA :  630
67_YZ127_T : ----------  ----------  ----------  ----------  ----------  ----------
---  :     -

ttgcatctgaaact gagcaacataaccat gaagagggagaggca ctgaattggtggggt aatatgtattatgtt gcataa

                          *       1300          *       1320          *       1340          *
1360
HDZCOMB     :
TCAAGATCAAGAACATTGTATAATTAGATACATCATGTAATTTGGGTGATTTGGGCGCCTAGGTGTTGAAATGTCTTTAA : 1356
34_YZ119_T : ----------  ----------  ----------  ----------  ----------  ----------
---  :     -
90_YZ218_M :
TCAAGATCAAGAACATTGTATAATTAGATACATCATGTAATTTGGGTGATTTGGGCGCCTAGGTGTTGAAATGTCTCTAA :  577
83_YZ211_M : ----------  ----------  ----------  ----------  ----------  ----------
---  :     -
85_YZ213_M :
TCAAGATCAAGAACATTGTATAATTAGATACATCATGTAATTTGGGTGATTTGGGCGCCTAGGTGTTGAAATGTCTCTAA :  572
66_YZ127_T :
TCAAGATCAAGAACATTGTATAATTAGATACATCATGTAATTTGGGTGATTTGGGCGCCTAGGTGTTGAAATGTCTCTAA :  710
67_YZ127_T : ----------  ----------  ----------  ----------  ----------  ----------
---  :     -
             t caagatcaagaacat tgtataattagatac atcatgtaatttggg tgatttgggcgcctag gtgttgaaatgtct
taa

                          *       1380          *       1400          *       1420          *
1440
HDZCOMB     :
GCCAAGCTTAGTAAAATAAAAAGTCCAGTAATTTGGGCAATGGCGTCCTTTGTTGTACT TAAAAAAAATTTATA ATAAAC : 1436
34_YZ119_T : ----------  ----------  ----------  ----------  ----------  ----------
---  :     -
90_YZ218_M : GCCAAGCTTAGTAAAATAAAAAGTCCAGTAATTTGGGCAATGGCGTCC----------  ----------
---  :  625
83_YZ211_M : ----------  ----------  ----------  ----------  ----------  ----------
---  :     -
85_YZ213_M : GCCAAGCTTAGTAAAATAAAAAGTCCAGTAATTTGGGCAATGGCGTCC----------  ----------
---  :  620
66_YZ127_T : GCCAAGCTTGGTAAAATAAAAAGTCC----------  ----------  ----------  ----------
---  :  736
67_YZ127_T : ----------  ----------  ----------  ----------  ----------  ----------
---  :     -
             g ccaagctt gtaaaa taaaaagtcc

                        *       1460          *       1480
HDZCOMB     : T ATTGAAGTTAAGCAA AAAAAAAAAAAAAAA AAAAAAAAAAA : 1 478
34_YZ119_T : ----------  ----------  ---------- :     -
90_YZ218_M : ----------  ----------  ---------- :     -
83_YZ211_M : ----------  ----------  ---------- :     -
85_YZ213_M : ----------  ----------  ---------- :     -
66_YZ127_T : ----------  ----------  ---------- :     -
67_YZ127_T : ----------  ----------  ---------- :     -
```

3.3.7 Sequence analysis by BLAST homology searches

The initial identification of the subtracted cDNA clones was confirmed using the extended, high quality sequence information derived from the RACE cloning for homology searches. Guided by the initial identification, the cDNA sequences were converted to amino acid sequences then were submitted to the BLASTP homology searching program. Information from BLASTP searching revealed the similarity of homologue proteins to each submitted sequences (the details were shown in Appendix).

The bZip sequence shows greatest similarity (38/76, 50% identities; 51/76, 67% positives) to a putative bZIP transcription factor in rice (Oryza sativa, japonica cultivar-group), and high similarity to RF2a (a bZIP transcription factor of rice), VSF-1 (a tomato bZIP transcription factor).

The PP2C sequence has extremely high similarity (252/283, 89% identities; 267/283, 94% positives) to Protein phosphatase 2C in *Mesembryanthemum crystallinum* (Common ice plant).

The LPL sequence exhibits greatest similarity (164/209, 78% identities; 182/209, 87% positive) to Lysophospholipase-like protein in *Arabidopsis thaliana*.

The cycMf2 sequence presents greatest similarity (144/175, 82% positive; 155/175, 88% positive) to soybean (*Glycine max*) mitotic cyclin a2-type, and high similarity to tobacco A-type cyclin, tomato cyclinA2.

3.4 Discussion

3.4.1 2,4-D induces cell division

The procedure for direct somatic embryogenesis can be divided into two main stages: induction and development. The induction stage involves the reactivation of cell division and acquisition of embryogenic competence. A large body of experimental data has indicated that reactivation of cell division is necessary to initiate the somatic embryogenesis developmental programme. Investigation of the expression of genes that

are components of normal cell functions such as cell division and stress responses could be an important strategy for the molecular study of somatic embryogenesis. These genes may reflect the reactivation of embryogenic cell division in differentiated alfalfa cells. Previous work in this laboratory has demonstrated that *Arabidopsis cdc2a* is a good marker for cell division competence and *CycB1;*1 is a suitable marker for cell division in alfalfa (Shao's, 2000). In my study, these gene promoters were used to investigate the role of 2,4-D in the activation of cell division during the early induction of somatic embryogenesis in alfalfa. In the experiment of different time course treatments by 2,4-D during the induction, *cdc2a* expression was detectable even after just 2 min 2,4-D treatment, with an increase in *cdc2a* expression following the increase in exposure time (Figure 3.6 and table 3.4). Almost no expression was detected for the 2,4-D-free treatment. This results indicated that 2,4-D is an effective signal to induce cell division competence in alfalfa. Unfortunately, information from *CycB1;*1 expression by very short 2,4-D treatment as described in *cdc2a* was absent due to the plants not being available at that time.

3.4.2 The role of 2,4-D during the induction of somatic embryogenesis in alfalfa

The influences of exogenously applied 2,4-D on the induction of somatic embryogensis have been well documented (for reviews, e.g., Dudits *et al.*, 1991; Yeung, 1995) and a growing body of recent experimental observations indicate that 2,4-D affects endogenous indoleacetic acid (IAA) and abscisic acid (ABA) level during the early phases of embryogenesis. Higher endogenous IAA concentration has been shown to be associated with an increased embryogenic response in various species/explants (Rajasekaran *et al.*, 1987; Ivanova *et al.*, 1994; Michalczuk and Druart, 1999; Jimenez and Bangerth, 2001 a, b, c). The experimental evidence from Senger's studies (2001) indicates the contribution of endogenous ABA to the induction phase of somatic embryogensis. Taking all of these observations together, one can hypothesise that the parallel induction of these pathways can lead to the morphological and developmental changes observed during the transition from somatic to embryogenic cell types due to rapid de- and re-dedifferentiation. 2,4-D appears to play the key role in stimulating the interaction between auxin and stress/ABA

signalling leading to cellular adaptation, genetic, metabolic and physiological reprogramming, which results in the embryogenic competence of somatic plant cells. The results in this study demonstrate that the duration of 2,4-D exposure necessary to reactivate cell division and induce embryogenic competence are different. One hour, 10 min, or even 2 min treatment with 2,4-D is sufficient to activate cell cycle gene expression. However, somatic embryos are not formed after transfer to development medium. The patterns of cell division competence and cell cycle gene *cdc2a* expression during the induction phase, and throughout the development phase, were found to be similar after 2 min treatment, or during continuous exposure to 2,4-D. However, the subsequent development of the globular clusters of dividing cells formed during the induction phase depends upon a more prolonged exposure to 2,4-D. In the system used in this study, embryos were only found after the complete induction period of 2,4-D treatment. Previous work in this laboratory has shown that two days induction is the minimum duration of 2,4-D treatment required for embryo formation (Shao, 2000), with an increased embryogenic tendency being exhibited in parallel with the extension of the 2,4-D treatment. Shorter than the minimum duration of 2,4-D treatment, e.g., 1 h treatment (Shao, 2000), 2 d treatment (Figure 2.10, D), resulted in root formation; between 1 h to 2 min treatment caused callus formation. These results indicate that the root organogenesis as an intermediate programme between callus and embryos, and appropriate induction treatment by 2,4-D is a key component for somatic embryogenesis in the later stages.

3.4.3 Different effects of 2,4-D in alfalfa and tobacco

In order to investigate the role of 2,4-D in the activation of cell division in different plant systems, three cell cycle gene promoters *cdc2a, CycB1;*1 and *CycA2* were involved in this study. The induction of direct somatic embryogenesis in alfalfa involves the re-activation of cell division in non-dividing (G0) leaf cells. In this study, the comparison of alfalfa and tobacco leaf explant suspension cultures in response to the standard procedure for the induction of direct somatic embryogenesis showed that the expression patterns of cell division genes in these two model systems are different. In alfalfa, the expression of *cdc2a*

and *CycB1;*1 was induced specifically by 2,4-D. Almost no gene expression was detected in leaf explants treated with 2,4-D-free induction medium. The expression of both genes (*cdc2a* and *CycB1;*1) was detectable from 2-3 days induction throughout the developmental stage and the embryos present in the late development period (Figure 2.7). *Cdc2a* expression was not restricted to dividing cells but was also exhibited in non-dividing cells. In tobacco, *cdc2a* expression was detectable after 2 days in the induction medium irrespective of whether 2,4-D was present or absent (Figure 3.1). The obvious expression of *cdc2a* was observed in the trichomes of tobacco explant (Figure 3.1, A, B, E and F). This result indicated that *cdc2a* expression in tobacco did not require 2,4-D, and could be stimulated by some other component of the induction system (kinetin, mechanical wounding etc.). Similar results have been demonstrated in other studies (Hemerly *et al.*, 1993). The analysis of *cdc2a* expression in tobacco leaf protoplasts derived from transgenic plants containing the *Arabidopsis cdc2a* promoter::*gusA* construct showed that transcription of the *cdc2a* gene was induced by either auxin or cytokinin but that it was not dependent upon the completion of DNA synthesis or progression through the cell cycle (Hemerly *et al.*, 1993). Thus *cdc2a* expression is not always coupled with cell proliferation but always precedes it, leading to the proposal that *cdc2a* expression may reflect a state of "competence to divide" (Hemerly, *et al.*, 1993). In the case of *CycB1;*1, different expression patterns were found in the two plant systems. In alfalfa, the expression of *CycB1;*1 was detectable after 3 days induction with 2,4-D and then throughout the remaining period. By 16 days induction, *CycB1;*1 expression showed a "spotty" pattern on explants detected by GUS staining, the expression being restricted to globular pro-embryos within the surrounding tissues (Figure 2.7). Results from previous work in this laboratory showed that *CycB1;*1 expression is strictly correlated with mitotic activity, and its expression is confined to dividing-cells (Shao, 2000). In tobacco, *CycB1;*1 expression also required the presence of 2,4-D and expression was detectable after 2 days 2,4-D induction. The expression was not detectable in 2,4-D free induction experiment or in the development period (Figure 3.3). Ferreira *et al.* (1994b) reported that in tobacco protoplasts, an increase in *CycB1;*1 (*cyc1*At) expression was observed only when cell division was induced. On this basis it was proposed that *CycB1;*1 (*cyc1*At) expression is restricted to dividing cells and that *CycB1;*1 (*cyc1*At) is involved in the regulation of the

G2 to M transition (Ferreira, *et al.*, 1994b). These observations indicated that cytokinin alone does not induce cell division in the tobacco explants. In addition, the fact that the expression of *CycB1;*1 in tobacco could not be maintained after transferring to development medium (Table 3.3) indicated that cell division did not occurred during this stage. This could explain, at least to some extent, why (unlike in alfalfa) very elongated cells were formed and no embryos were produced during the development stages of tobacco experiments in this study. The exact reasons why the cell division stopped in these stages are unclear. It has been demonstrated that the minimum concentration or duration of 2,4-D treatment required for an inductive effect is different in various genotypes and species (Dudits *et al.*, 1991, 1995). In some plant systems, very high concentrations of 2,4-D are required for somatic embryo induction, e.g., in cultures of cotyledons of *Serenoa repens*, induction with 2,4-D at 452 μM was found to be effective (Gallo-Meagher and Green, 2002).

The expression pattern of *CycA2* in tobacco was found to be substantially different from that of *CycB1;*1 and was more similar to that of *cdc2a*. *CycA2* appears to be a typical A-type cyclin and is expressed earlier than *cyc1*At, appearing in the S phase as well as G2 and early M phase (Shaul *et al.*, 1996).

Based on the comparison of the expression patterns of these cell cycle gene promoters in the two plant systems, it can be proposed that the role of 2,4-D in the activation of cell division is different in different plant systems. 2,4-D treatment resulted in different expression patterns of the cell cycle gene promoters in these two plants, indicating that different cell division patterns were activated in the two systems.

3.4.4 Gene expression during the early stages of induction of somatic embryogenesis

The resetting of the whole ontogenic programme by initiation of somatic embryogenesis requires an essential reprogramming of gene expression patterns. One of the basic features in initiation of somatic embryogenesis is the reactivation of the cell cycle in differentiated plant cells under the influence of external stimuli, eg., 2,4-D treatment. The artificially induced series of cell divisions opens the way to switch from a somatic to embryogenic cell type that requires coordinated expression of sets of genes and the post-translational

modifications of the regulatory proteins involved in embryogenesis. Based on these considerations, molecular studies on somatic embryogenesis may be focused on the regulatory mechanisms in cell cycle with special emphasis on the mode of signal transduction (Dudits *et al.*, 1995). In my investigation, "mini-array" dot blot hybridization was used to determine the expression patterns of some 100 cDNA clones isolated by subtractive hybridization during the early stages of somatic embryogenesis. mRNA isolated from different time course treatments during the induction of somatic embryogenesis in alfalfa leaves was used to prepare the probes. Results from this work indicated that most of the cDNA clones are induced after 7 days induction by 2,4-D treatment during the early stages of somatic embryogenesis (Table 3.1). This observation agreed with the data obtained from previous work (Shao's PhD thesis 2000). Some genes were activated following a very short 2,4-D treatment (2 min or 10 min treatment), including EP1 RecPK, PG inhibitor (protein kinase), PMM (post-translational protein), Endochitinase class I, Endo-1,3-β-glucosidase (stress induced protein), ADH (redox enzyme), RSI-1b (developmental regulatory protein), and two unknown proteins ("GluRich", "Y hyp"). It is noticeable that several of the genes that are induced by very short exposure to 2,4-D are stress-response genes, even though these genes are expressed later than the cell growth-related genes such as the ribosomal protein genes during a continuous 2,4-D induction treatment (Shao, 2000).

In this study, much higher levels of expressions of the ribosomal protein group genes were observed after 7 days induction. Similar results were also found in Shao's study (PhD thesis, 2000) in which this group of genes showed significant levels of expression after 3 days induction and reached a peak on day 5. The rapid induction of ribosomal proteins by 2,4-D indicates that one of its functions is as a stimulus for cell growth and protein synthesis. Data from the present study showed that no detectable expression of the group of ribosomal protein genes 7 days after one hour of 2,4-D treatment was observed (table 3.1). These observations demonstrated that short exposure to 2,4-D (e.g., 2 or 10 min) results in stress gene expression, while the expression of cell growth genes (such as ribosomal protein genes) requires longer 2,4-D induction treatment. This conclusion is consistent with the proposal that 2,4-D has a dual role in the induction of somatic

embryogenesis- as an auxin and as an inducer of stress responses (Fehér *et al.*, 2001, 2002) (Figure 3.12).

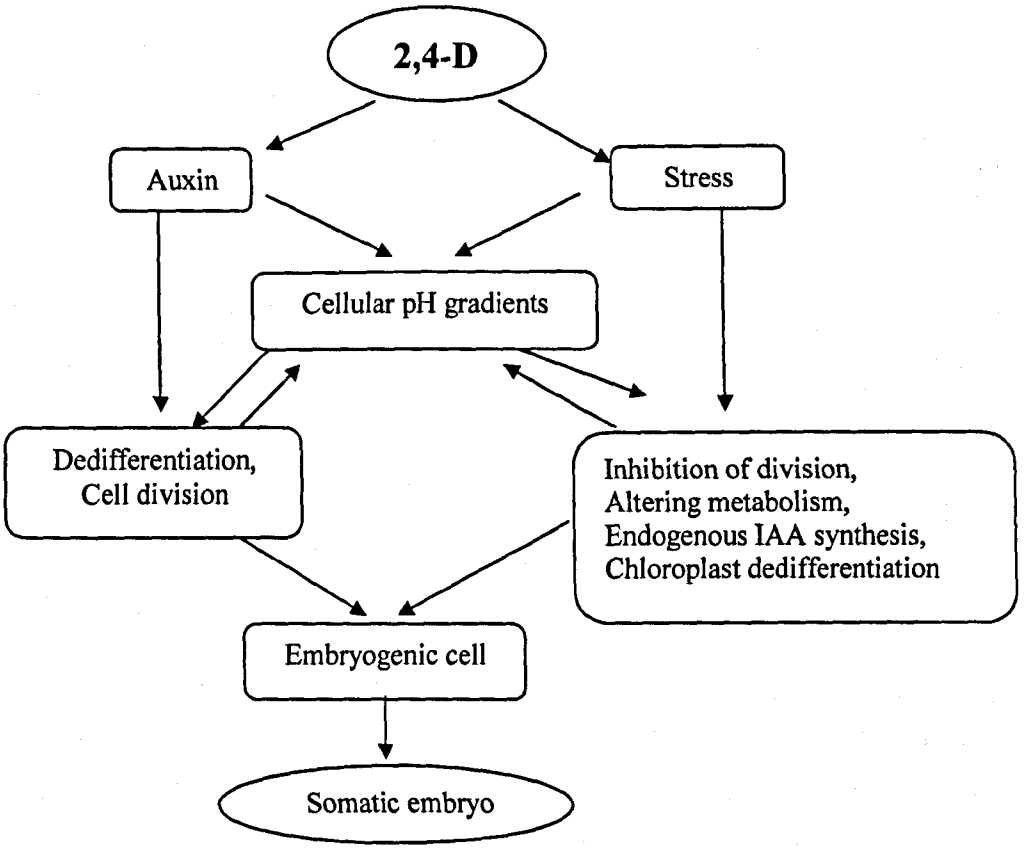


Figure 3.12. The model of dual effect by 2,4-D treatment during the induction of somatic embryogenesis (modified from Fehér *et al.*, 2001).

Two of the embryogenesis-related genes were identified as homologues of the auxin-induced proteins IAA7 and IAA9 from *Arabidopsis thaliana*. These proteins have been proposed to mediate tissue-specific and cell-type restricted responses to auxin during plant growth and development (Abet *et al.*, 1995). In Shao's study (PhD thesis, 2000), the expression of IAA7 and IAA9 increased significantly between 3-5 days and results from my study showed that high levels of expression of these two genes could be observed after 7 days induction, suggesting that these two genes may be related to the early stages of

induction of embryo development. Several lines of evidence from other studies appear to support this view. In alfalfa leaf protoplasts cultured in the presence of 2,4-D, the endogenous IAA levels increased considerably during the first 2-3 days of culture (Pasternak *et al.*, 2002). A similar peak of endogenous IAA level has been observed in immature zygotic sunflower embryos induced to form somatic embryos (Charriére *et al.*, 1999). In the sunflower system, the tissues grown under embryogenic conditions showed a 4-fold increase in their IAA content as compared to those tissues that followed the caulogenic pathway. Immuno-cytochemical localization of IAA in the immature zygotic embryos before, during and after the induction of somatic embryo development provided direct evidence that an endogenous auxin pulse may be one of the first signals leading to somatic embryogensis (Thomas *et al.*, 2002). Based on these observations, the level of endogenous IAA could be considered as marker for auxin response and potential of somatic embryogenesis.

3.4.5 Analysis of the selected clones

Several clones of interest, which encode regulatory components, such as transcription regulators (HD-Zip, bZIP), protein kinases/phosphatases (PP2C and LPL) or cell division related factor (cycMf2), were selected for further analysis, because these genes possibly involve the regulatory mechanism of somatic embryogenesis process.

Transcription factors play an important role in the regulation of gene expression due to their ability to bind to specific DNA sequences and to activate transcription (Torres-Schumann *et al.*, 1996). They can interact with other proteins involved in transcription, change their properties upon modification (Hunter and Karin, 1992) and, in some cases, respond directly to external stimuli (Stone and Sadowski, 1993). Several classes of transcription factors have been defined on the basis of common amino acid sequence motifs. bZIP proteins contain a basic domain followed by a leucine zipper motif with a heptad repeat of leucines (Landshulz *et al.*, 1988). bZIP proteins are the most abundant class of transcription factors cloned from plants. Their possible contribution to regulatory processes as diverse as the tissue-specific expression of zein genes (Schmidt *et al.*, 1992), the light responsive expression of chalcone synthase genes (Weisshaar *et al.*, 1991), gene

expression in response to ABA (Guiltinan *et al.*, 1990) and viral gene expression (Katagiri *et al.*, 1989) indicate that bZIP proteins are of widespread importance in controlling plant development. Functional analysis *in vivo* demonstrated the importance of some of the cloned bZIP proteins in the activity of promoters containing the corresponding specific recognition sequence.

HD-Zip proteins include members that contain a homeodomain/leucine-zipper (HD-Zip) motif (see chapter 4). bZIP and HD-Zip transcription factor genes investigated in this study have shown that their expression required a prolonged 2,4-D induction (5-7 days). These results may help explain, to some extent, why there is no embryo found or very poor embryogenesis in the case of less than three days 2,4-D induction in previous studies. In other studies, the regulation of homeobox-containing genes during somatic embryogensis was reported to be in close association with the early development of embryos in carrot (Kawahara *et al.*, 1995) and soybean (Ma *et al.*, 1994). The expression of plant homeobox genes also suggested that this type of transcription factor controlled cell differentiation associated with vascular tissue formation and development (Ma *et al.*, 1994; Tornero *et al.*, 1996). Results from WU-BLASTp searching revealed that the alfalfa bZIP gene characterised in this study has the greatest similarity to a putative bZIP transcription factor in rice (*Oryza sativa*, japonica cultivar-group), and high similarity to RF2a (a bZIP transcriptional activator of phloem-specific gene expression in rice) (Yin *et al.*, 1997) and to tomato bZIP transcription factor VSF-1 (Ringli and Keller, 1998). Both RF2a and VSF-1 are involved in the control of vascular development. In this respect, it is of interest that the first cells to re-enter the cell cycle in the alfalfa leaf explants are in the vasculature.

The control of many biological processes is mediated by the reversible phosphorylation of proteins catalysed by protein kinases and phosphatases. Eukaryotic organisms frequently encounter environmental conditions that cause cytotoxic damage, hence they have developed sophisticated systems for sensing and responding to physiological stress. Protein kinase cascades are at the core of these stress sensor pathways. Recent studies suggest that stress-activated kinase cascades are negatively regulated by two types of protein phosphatases: tyrosine-specific enzymes and serine-threonine phosphatases of the type 2C class (PP2C) (Takekawa *et al.*, 1998; Gaits *et al.*, 1997). Studies of PP2C are

consistent with a role for PP2C in the regulation of a Ca^{2+}-dependent signal transduction system. Ca^{2+} activates kinase cascades primarily by binding to its intracellular receptor, calmodulin (CaM), and to the regulatory domain. The Ca^{2+}-CaM-dependent kinases (CaM kinases) are activated by binding to Ca^{2+}-CaM but also depend upon phosphorylation. These stimulatory phosphorylation events seem to be the combined result of phosphorylation by upstream CaM-kinase, and Ca^{2+}-CaM-induced autophosphorylation (Millward *et al.*, 1999). The results from dot blot hybridization in this study demonstrate that PP2C, calmodulin and lysophospholipase could all play important roles during the induction of somatic embryogensis.

Cyclins are very important regulatory elements involved in the control of cell division. Sequence analysis and WU-BLAST2 results (see Appendix) revealed that cycMf2 belongs to the plant group A-type cyclins. CycMf2 sequence shows the greatest similarity to soybean mitotic cyclin A2-type. Most A-type cyclins are expressed from S-phase through to mitosis (can be earlier in plants) (Fowler *et al.*, 1998b).

DNA sequencing results of this study showed that the RACE reactions were generally successful. Good quality sequence data were eventually obtained from the 3' RACE products of all four genes (bZip, PP2C, lysophospholipase and cyclin Mf2) (Figure 3.7-3.10). However, the 5'RACE products appeared to be more difficult to clone and sequence. As a result, there is no upstream sequence information for the bZip or cycMf2 genes. The 5' RACE sequence for the PP2C gene overlaps with the original cDNA clone but does not extend to the start of the mRNA sequence. The 5' RACE product of the lysophospholipase gene failed to be sequenced to the primer sequence in the original cDNA clone. It is supposed that there is an existing "floating" section of 5' sequence upstream of the cDNA clone. The problems with cloning and sequencing the 5' RACE products may be due to poor quality of DNA samples, but the exact reasons are not clear.

In addition, the results from this study also revealed that there are many interesting genes which could be related to the induction of the embryos formation, e.g., *shaggy*-related protein kinase, cytoskeletal factors, RSI1, etc. Homologues of *shaggy* have been cloned from many organisms and shown to be involved in signal transduction pathways that control patterning, cell fate determination and cytokinesis (Pay *et al.*, 1993; Bogre *et al.*, 1995; reviewed by Welsh *et al.*, 1996), which is of central importance to the

understanding of plant development. RSI1 has been identified as a molecular marker for lateral root initiation in tomato (Taylor and Scheuring, 1994). In transgenic tomato seedlings containing a RSI1 promoter::*gusA* fusion , GUS activity was observed in both lateral and adventitious root initials, including very early initials, and lasted until shortly after the lateral emerged from the parent tissue. In roots from seedlings with high activity, GUS expression was also observed in the root cap and vascular tissue (Taylor and Scheuring, 1994). The investigation in our laboratory suggested that the RSI1 gene may be a suitable marker of somatic embryo induction, because it is activated rapidly after alfalfa leaf pieces are cultured in induction medium and reaches a peak after 3-5 days induction (Shao's PhD thesis, 2000), remains high through to day 7 (in this study). Interestingly, comparing with RSI1, RSI-1b exhibited a different expression pattern; it was activated very shortly after 2,4-D treatment (2 min treatment, data not shown) and reached a peak with 7 days induction treatment (in this study). This finding suggested that RSI-1b could be involved in the very early stages of initiation required for embryogenic reprogramming.

It is clear that this investigation has exhibited intriguing results, in which there are many interesting genes that are activated during the induction of somatic embryogenesis. Mostly they are stimulated and substantially expressed after 7 days of induction, suggesting very important roles of these genes for the induction or formation of embryos. Some of them are even stimulated by very short (2 min or 10 min) induction treatment, suggesting that these "early stages"-expressed genes could play important roles during the very early events of somatic embryogenesis and further progression leading to the embryogenic pathway. There is no doubt that further analysis of these clones will generate more information that will contribute to understanding the relationship between temporal expression of these genes and their functions during embryogenesis.

CHAPTER 4 THE ROLE OF THE HD-Zip TRANSCRIPTION FACTOR *Mfhb-1* IN SOMATIC EMBRYOGENESIS

4.1 Introduction

In this study, a gene encoding a transcription factor of the homeodomain/leucine zipper (HD-Zip) class was identified in the subtractive cDNA library. This gene was chosen for further investigation, because similar genes have been associated with somatic embryogenesis in other systems. The first stage of this investigation was to obtain the full sequence of the gene. In order to further characterise the biological functions of this HD-Zip trancription factor gene during somatic embryogenesis in alfalfa, sense and antisense transformation techniques were then used to introduce fragments of the gene into transgenic alfalfa plants and determine the effect on somatic embryogenesis.

4.1.1 Transcription factors

The phenotypic differences that distinguish the various kinds of cells in a higher eukaryote are largely due to differences in the expression of genes that code for proteins, that is those transcribed by RNA polymerase II. In eukaryote systems RNA polymerase II principally transcribes DNA to mRNA, which in turn is translated to produce protein at the ribosomes. Because RNA polymerase II cannot recognise target promoter sequences or respond to developmental or environmental signals, transcription is normally mediated by the formation of a complex between RNA polymerase II and a number of DNA binding proteins termed transcription factors. There are a number of general transcription factors termed initiation factors that are required for all transcriptional activity, designated TFIIB, TFIID, TFIIE, TFIIF and TFIIH (Nikolov and Burley, 1997). In addition to these general initiation factors, the response of a gene to developmental and environmental factors is controlled by a group of transcription factors comprising accessory factors, transcriptional activators and co-activators. The functional complex consisting of the RNA polymerase and transcription factors assembles upon the promoter region of the target DNA region and transcribes the DNA from 5' to 3' until a termination sequence is

detected by the RNA polymerase complex. The resultant mRNA is then released to ultimately produce a biologically active protein.

4.1.2 Transcription factors in higher plants

In recent years, tremendous experimental effort has led to the identification of transcription factors *in vitro* in human, rat, *Drosophila*, and yeast. Although many transcription factors have been isolated from plants the degree of characterisation for the majority of them has been less extensive than in animal and yeast systems (Schwechheimer *et al.*, 1998). However, it has been possible to characterise functional domains *via* comparison of amino acid sequences with previously characterised animal and yeast analogues (Liu *et al.*, 1999). Despite a considerable degree of homology between most classes of eukaryotic transcription factors, there are some novel functional groups within plant transcription factors that do not occur in non-plant systems. Study of functional domains in plants by mutational and functional analysis has revealed that typical plant transcription factors consist of a DNA binding region, an oligomerisation site, a transcription regulation domain and a nuclear localisation signal (NLS) although some proteins lack either a transcription regulation domain or a DNA binding region (Liu *et al.*, 1999).

The DNA binding region of the transcription factor has the ability to form a complex between certain amino acid residues and DNA at *cis* acting elements. The *cis* acting elements provide the selectivity to the DNA binding of the transcription factor. DNA binding is further enhanced through the non-specific association of a number of amino acids with either phosphate or deoxyribose moieties (Liu *et al.*, 1999). The base recognition sequences within the transcription factors are highly conserved in order to retain their sequence specificity.

For many transcription factors, activation, inactivation or DNA binding specificity is associated with protein interaction such as heterodimerisation or protein kinase mediated modification (Schwerchheimer *et al.*, 1998). These specific protein interactions occur at the oligomerisation domains. The conformational changes associated with binding may activate, inactivate or change the properties and even the function of the protein. These

changes may have a direct impact on any combination of the following: DNA binding specificity, promoter element recognition and nuclear localisation (Liu *et al.*, 1999). Transcription factors can form homo-oligomers (through binding with identical proteins) and also hetero-oligomers (through binding with different proteins). Like the specific DNA binding regions, the oligomerisation domains are also highly conserved and therefore failure to bind correctly would clearly affect function.

The transcription regulation-domains are the regions within transcription factors that control transcription. The regulation can be either repression or activation of transcription, depending upon whether the transcription factor inhibits or stimulates transcription respectively.

The plant transcription factors have been classified into families according to similarities with other eukaryotic transcription factors. Thus, they have been characterised with respect to either their structural features within the most highly conserved region (e.g. the Zinc finger family that all have cysteine and/or histidine residues associated with a zinc ion) or by structural domains which lie outside the most highly conserved region (e.g. the homeodomain family that have an amino acid sequence approximately 60 residues in length producing either 3 or 4 α-helices and a N-terminal arm) (Liu *et al.*, 1999). Table 4.1 shows the molecular structures that have been used to classify transcription factor domains within plants.

Domain type	Structure	Reference
Zinc finger	Finger motif(s) each maintained by cysteine and/or histidine residuces organised around a zinc ion.	Sakamoto *et al.*, 1993.
bZIP	A basic region and a leucine-rich zipper-like motif.	Mikami, *et al.*, 1994.
Myb-related	A basic region with one to three imperfect repeats each forming a helix-helix-turn-helix.	Ogata, *et al.*, 1994 ; Wang, *et al.*, 1997.
Tri-helix Basic	Acidic and proline/glutamine-rich motif which forms a tri-helix DNA-binding domain.	Kuhn, *et al.*, 1993; Ni, *et al.*, 1996.
Homeodomain	Approximately 60 amino acid residues producing either three or four α-helices and an	Schindler, *et al.*, 1993; Watillon, *et al.*, 1997.

	N-terminal arm.	
Myc b/HLH	A cluster of basic amino acid residues adjacent to a helix-loop-helix motif.	Sainz, *et al.*, 1997.
MADS	Approximately 57 amino acid residues that comprise a long α-helix and two β-strands.	Pellegrini, *et al.*, 1995.
AT-hook motif	A consensus core sequence R (G/P) RGRP with the RGR region contacting the minor groove of A/T-rich DNA.	Gupta, *et al.*, 1997.
HMG-box	l-shaped domain consisting of three α-helices with an angle of about 80° between the arms.	Grasser, *et al.*, 1995.
AP2/EREBP	A 68-amino acid region with a conserved domain that constitutes a putative amphiphatic α-helix.	Jofuku, *et al.*, 1994.
B3	A 120 amino acid conserved sequence at the C-termini of VP1 and AB13.	Suzuki, *et al.*, 1997.
ARF	A 350 acid region similar to B3 in sequence.	Guilfoyle, *et al.*, 1998; Ulmasov, *et al.*, 1997.

Table 4.1 Structural features of conserved domains that are used to classify plant transcriptionfactors. These domains, the most conserved regions within genes of the same family, are responsible for DNA-binding and oligomerization. (From Liu *et al.*, 1999)

4.1.3 Homeodomain transcription factors in higher plants

The homeo-domain (HD) is an approximately 60 amino acid sequence encoding the homeobox (HB), the sequence and structure of which are highly conserved in animal, fungal and plant proteins (see Bürglin, 1994, for review). The homeobox is a DNA sequence motif that was first discovered in genes associated with a number of developmental (homeotic) mutations in *Drosophila*. The homeobox has since been recognised in many animal systems. Ample evidence has now accumulated that these genes control many crucial developmental steps within multicellular eukaryotic systems. The homeobox gene products are thought to act as molecular switches to determine cell fate within developing cells by directly modulating gene expression (Schena and Davis,

1992). The homeodomain provides site-specific DNA binding capability and the mechanism of binding has been studied both structurally and genetically, providing the following general model. It is suggested that helix 3 (the most highly conserved of the three α-helices) fits directly in to the major groove of the DNA structure (Schena and Davis, 1992). Although much less is known about the function of plant homeobox genes, accumulating evidence also points to a central role in developmental control. Some plant homeobox genes appear to be linked to developmental mutant phenotypes, such as maize *KNOTTED-1* (Vollbrecht *et al.*, 1991), barley *HOODED* (Müller *et al.*, 1995), *Arabidopsis GLABRA2* (Rerie *et al.*, 1994), *BELL1* (Reiser *et al.*, 1995) and *SHOOTMERISTEMLESS* (Long *et al.*, 1996). A number of homeobox genes in *Arabidopsis* have been identified as regulators of shoot apical meristem (SAM) formation and maintenance, such like *CUP-SHAPED COTYLEDON 1* and *2* (*CUC1* and *2*) (Aida *et al.*, 1997; Takada *et al.*, 2001), *SHOOT MERISTEMLESS* (*STM*) (Barton and Poethig, 1993) and *WUSCHEL* (*WUS*) (Mayer *et al.*, 1998). Despite a number of differences between plant and animal developmental systems, the basic transcriptional regulatory mechanisms remain highly conserved. This suggests that underlying regulation principles may be similar in animal and higher plant systems (Schena and Davis, 1992). Therefore it is possible to compare animal systems with those of higher plants enabling known animal mechanisms to be applied to analogous higher plant mechanisms.

4.1.4 HD-Zip transcription factors

The homeodomain-leucine zipper (HD-Zip) proteins constitute a family of putative transcription factors that appears to be unique to higher plants. The HD-Zip proteins differ from other HD proteins in that they contain a leucine zipper motif closely linked to the carboxy-terminal end of the HD region. *In vitro* studies have revealed that the HD-Zip proteins are able to dimerise *via* their leucine zipper motifs and that dimerisation is a prerequisite for DNA binding (Johanson *et al.*, 2001; described by Hanson *et al.*, 2002). Further studies have shown that some HD-Zip proteins have the ability to dimerise with other HD-Zip proteins producing hetero-dimers that could possibly have a different cellular function (Frank *et al.*, 1998; quoted by Hanson *et al.*, 2002).

The HD-Zip family was first discovered in maize (Vollbrecht *et al.*, 1991) and *Arabidopsis thaliana* (Ruberti *et al.*, 1991; Schena and Davis, 1992). The HD-Zip genes isolated from the genome of *Arabidopsis thaliana* have shown that the HD-Zip family has over 25 members that are divided in to subfamilies. The HD-Zip transcription factor subfamilies were originally named HAT1, HAT2, ATHB8 and GL2, these were later renamed HD-Zip I, HD-Zip II, HD-Zip III and HD-Zip IV respectively. The function of the HD-Zip genes is diverse both among different subfamilies and within each subfamily (Sakakibara *et al.*, 2001).

Because of the exclusive occurrence of the HD-Zip family in plants, members of this class of proteins may function in regulating certain aspects of development that are specific for plants, such as phenotypic responses to environmental signals (Schena and Davis, 1992). This idea is supported by studies on the HD-Zip families of *Arabidopsis*. The function of the HD-Zip I and II subfamilies are likely to be involved with signal transduction in relation to light, dehydration-induced ABA or auxin, i.e. transduction networks involved with growth regulation in plants (Sakakibara *et al.*, 2001). The overexpression of sense and antisense HD-Zip I and HD-Zip II genes generally affects the growth rate and development of plants (Schena *et al.*, 1993; Aoyama *et al.*, 1995; Meijer *et al.*, 1997; as described by Sakakibara *et al.*, 2001). Examples of characterised HD-zip genes include the ATBH-10, ATBH-2 and REVOLUTA genes (Hanson *et al.*, 2002).

The alfalfa HD-Zip gene isolated by subtractive cloning during somatic embryogenesis most closely resembles the *Arabidopsis* ATHB-1 gene. The *Arabidopsis* gene encodes a homeodomain leucine zipper class I transcription factor. It is expressed throughout all plant tissue but to a higher degree within the root and flowers. The expression of the ATHB-1 gene was rapidly induced by wounding, flooding and ethylene treatment. The increased expression as a response to ethylene corresponded to observations that ATHB-1 levels in the leaf increased with age and the highest levels were observed during bolting. The ectopic expression of the ATHB-1 gene in transgenic tobacco led to the conclusion that the ATHB-1 transcription factor is associated with leaf development (Aoyama *et al.*, 1995). The ATHB-1 gene altered leaf shape in transgenic *Arabidopsis* (Lucchetti *et al.*, 2001). In addition to these examples, other studies have found that some HD-Zip genes have roles related to the actions of plant hormones or the response of plants to

environmental factors (Carabelli *et al.*, 1993; Baima *et al.*, 1995; Soderman *et al.*, 1996, 1999; described by Hanson *et al.*, 2002). There are indications that other HD-Zip proteins may function in the regulation of secondary vascular development (Tornero *et al.*, 1996) and in the control of gene expression during embryogenesis (Kawahara *et al.*, 1995).

4.1.5 Sense and antisense technology to characterise transcription factors in higher plants

Two principal methods have been used to study transcription factors in transgenic plants; over-expression (sense) and antisense expression technologies. Over-expression requires the transformation of a plant with a construct containing the transcription factor of choice coupled to a strong constitutively expressed promoter such as the Cauliflower Mosaic Virus 35S promoter. The resultant plants will grow with a high level of transcription factor expression throughout the plant. It is also possible to use a strong tissue specific promoter, this will result in high level expression of the transcription factor in a particular area of the plant. If expression occurs in a cell or tissue where the gene is normally inactive, this is referred to as "ectopic expression". Anti-sense technology involves the insertion of the selected transcription factor gene in an inverted orientation i.e. the 5' terminal is at the 3' terminal and consequently the resultant antisense RNA will exhibit exact sequence complementary to the original transcription factor mRNA. The underlying mechanism is now thought to involve RNA interference (RNAi) interactions to effectively knockout the host analogue of the transcription factor, therefore the resultant plant will grow with a reduced level or complete absence of the transcription factor in question. The RNAi model as discussed by Sharp (2001), proposes that double stranded RNA molecules (i.e. the result of sense and anti-sense analogues binding through sequence complementary) are non-specifically processed to form RNAs 21-23 nucleotides in length, termed short interfering RNAs (siRNAs). SiRNAs direct the cleavage of mRNA through sequence homology, and are thus responsible for post translational gene silencing (Sharp, 2001).

Over-expression and anti-sense technologies are therefore powerful tools in determining transcription factor function. However, there are limitations to this method in that it is

difficult to assess the extent of over-expression or suppression within the plant. In addition, in over-expression systems it is difficult to determine whether the up-regulation of other genes is a direct consequence of the over expressed transcription factor, or occurs indirectly as a consequence of the expression of secondary transcriptional regulatory genes.

Anti-sense technology targets specific regions of RNA and this makes it possible for RNAi interactions to affect not only the transcription factor of choice but also any transcription factors that may share a conserved region. For example if anti-sense technology was employed against a transcription factor that contained a highly conserved region such as the homeodomain, then the post transcriptional gene silencing might not only effect the individual transcription factor of choice but other members of the homeodomain family.

In this study, the function of an alfalfa HD-Zip transcription factor gene was investigated. A partial cDNA clone of this gene was isolated and identified in the NBI laboratories by subtractive cloning. It is expressed during the early induction period in the alfalfa direct somatic embryogenic system (Shao, 2000). Sequence analysis of the partial cDNA sequence by BLAST homology search showed that the gene has the greatest sequence similarity to the *Arabidopsis* ATHB-1 HD-Zip transcription factor. For this reason, it was termed MFHB-1.

As a result of the RACE cloning and sequencing described in this chapter, the full length alfalfa HD-Zip transcription factor gene was shown to be about 1.3 kb in length and to include a uORF region upstream of the HD-Zip coding sequences. In order to investigate the biological role of this HD-Zip transcription factor gene during somatic embryogenesis in alfalfa, constructs containing various HD-Zip gene fragments (uORF, HD-Zip core sequence and the full length HD-Zip gene) in sense and antisense orientation were introduced into alfalfa plant tissue by *Agrobacterium*-mediated transformation procedure. The transgenic plants carrying these constructs were used to investigate the role of this HD-Zip gene in somatic embryogenesis.

4.1.6 pBECK400/6 as donor vector used in the construction

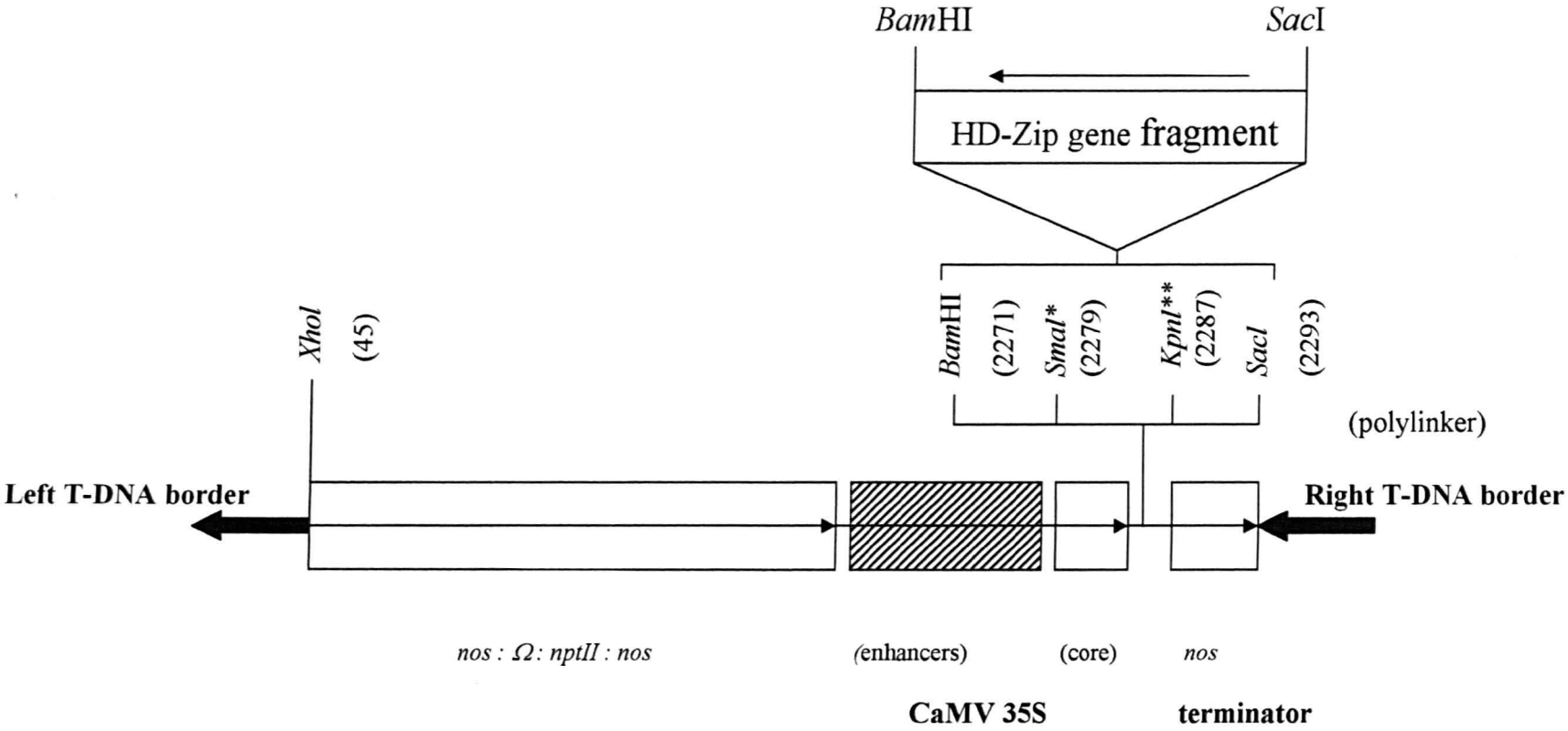
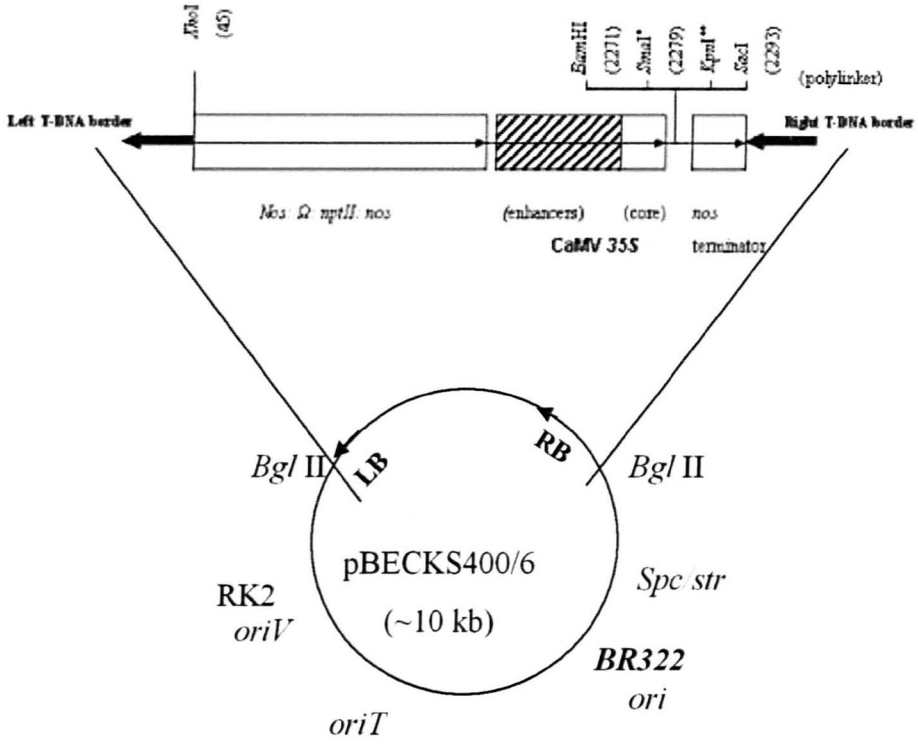


Figure 4.1A Schematic map of different HD-Zip gene fragment inserts in pBECKS400/6.



pBECKS400/6

Figure 4.1B Schematic map of pBECKS400/6

The pBECKS400/6 vector used in this study is one of a series of binary T-DNA vectors (pBECKS), which were developed by Dr A McCormac in the NBI laboratories (McCormac *et al.*, 1997) (see Chapter 2). pBECK400/6 was designed with a view to facilitating the insertion of novel sequences for transfer to plants (Figure 4.1A). The details of the construction of pBECK400 vector is shown in Figure 4.1B (McCormac *et al.*, 1997).

4.2 Materials and Methods

4.2.1 Preparation of donor vector used for the construction

The donor vector used for construction was pBECKS400/6 (McCormac *et al.*, 1997) under the control of the CaMV35S promoter, carrying the *spc/str* selectable marker gene on the backbone, conferring spectinomycin resistance property to the host bacterium.

4.2.1.1 Transformation of pBECKS400/6 with XL10-Gold Cell

XL 10-Gold Kan ultracompentent cells (supplied by Stratagene) were thawed on ice. After gently mixing by hand, a 40 µL aliquot of the cells was transferred into a chilled, 15 mL, Falcon 2059 polypropylene tube and 1.6 µL of the XL 10-Gold ß-mercaptoethanol mix provided with the kit was added. The contents of the tube was swirled gently and incubated on ice for 10 minutes with gently swirling every 2 minutes. Two µL of the ligating reaction (or plasmid pBECKS400/6) was mixed with the transformation reaction then swirled gently. The tubes were incubated on ice for 30 minutes. The tubes were given a heat pulse in a 42°C water bath for 30 seconds and then incubated on ice for 2 minutes. 0.45 mL of preheated (42°C) NZY+ broth was added to each tube and they were then incubated at 37°C for 1 hour with shaking at 225-250 rpm. 150 µL of each experimental transformation reaction was plated onto LB-spectinomycin agar plates using a sterile spreader. The plates were incubated at 37°C overnight.

4.2.1.2 Colony selection and subculture

Four to six single white colonies were chosen and inoculated into tubes containing LB-spectinomycin (50 mg L^{-1}) liquid medium individually. The suspension cultures were incubated at 37°C overnight with shaking (~150rpm)

4.2.1.3 Plasmid DNA isolation

The suspension cultures were processed to plasmid DNA isolation using the Wizard *Plus* Minipreps DNA Purification Kit (Promega) or Qiagen Minipreps DNA Purification Kit (Qiagen). The procedures were carried out according to the manufacturer's instructions. The concentration of plasmid DNA samples was determined by measuring the A_{260} in a UV spectrophotometer.

4.2.1.4 Confirmation of the transformation by restriction digestion

One μg pBECKS400/6 plasmid DNA from each sample was used to carry out restriction enzyme digestion with *Bam*HI and/or *Sac*I, to confirm successful transformation. The restriction enzyme digestion was conducted in a total volume of 20 μL including 2 μL MC-buffer, 0.2 μL BSA, 0.5 μL *Bam*HI and 0.5 μL *Sac*I (both *Bam*HI and *Sac*I supplied by Promega). The digest reactions were incubated at 37°C overnight and the products were analysed by agaraose gel electrophoresis.

4.2.2 Generation of the inserts

The full length HD-Zip transcription factor gene isolated by subtractive cloning and extended by RACE PCR was found to contain a highly conserved upstream open reading frame (uORF). The full length HD-Zip sequence was subcloned into the pGEMT-easy vector and several clones were re-sequenced for comparison with the original sequence. The $Y_{2\text{-}5}$ plasmid contained the correct sequence of the full length HD-Zip gene and was chosen to be used as template to amplify DNA fragments of the gene for insertion into transgenic alfalfa plants. The fragments were amplified with primers containing *Bam*HI and *Sac*I cohesive ends to facilitate construction of the following: a). full length HD-Zip

construct in sense orientation, b).the HD-Zip gene coding sequence only in both sense and antisense orientation and c). the uORF region in sense orientation. The PCR was performed in a volume of 50 μL consisting of 1 x PCR buffer, the four deoxyribonucleotides (100 μM each), the two relevant primers (1 μM each), 0.5 unit of *Taq* DNA polymerase and 1 μL of plasmid DNA solution.

4.2.2.1 The primer pairs and the relevant PCR cycle parameters

Fragment 1 - sense full length HD-Zip gene amplification:
forward primer (*Bam*HI) 5'-GGATCCCCTCCGGTAAACCCTTCAGTTC-3' and reverse primer (*Sac*I) 5'-GAGCTCGGACGCCATTGCCCAAATTAC-3'. The reaction was performed at 94°C for 3 min followed by 35 cycles of 94°C for 45 s, 60°C for 30s, and 72°C for 1 min and 45 s, and a final extension at 72°C for 3 min.
Fragment 2 - sense HD-Zip gene amplification:
Forward primer (*Bam*HI) 5'-GGATCCTTGTAGCACAACCTTTAACGTGAA-3' and reverse primer (*Sac*I) 5'-GAGCTCGGACGCCATTGCCCAAATTAC-3'; the PCR cycle parameters were as in 1)
Fragment 3 - antisense HD-Zip gene amplification:
forward primer (*Sac*I) 5'GAGCTCTTGTAGCACAACCTTTAACGTGAA-3' and reverse primer (*Bam*HI 5'-GGATCCGGACGCCATTGCCCAAATTAC-3'). The PCR reaction parameters were as in 1).
Fragment 4 – sense uORF fragment amplification:
forward primer (*Bam*HI) 5'-GGATCCCCTCCGGTAAACCCTTCAGTTC-3' and reverse primer (*Sac*I) 5'-GAGCTCCTTCACGTTAAAGGTTGTGCTACA-3'. The PCR reaction was performed at 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 2 min.
The PCR fragments were headed with *Bam*HI and ended with *Sac*I to allow cloning into the pBECKS400/6 vector in the correct orientation. The fragment length of HD-Zip gene and primers used were shown on table 4.2 and Figure 4.2.

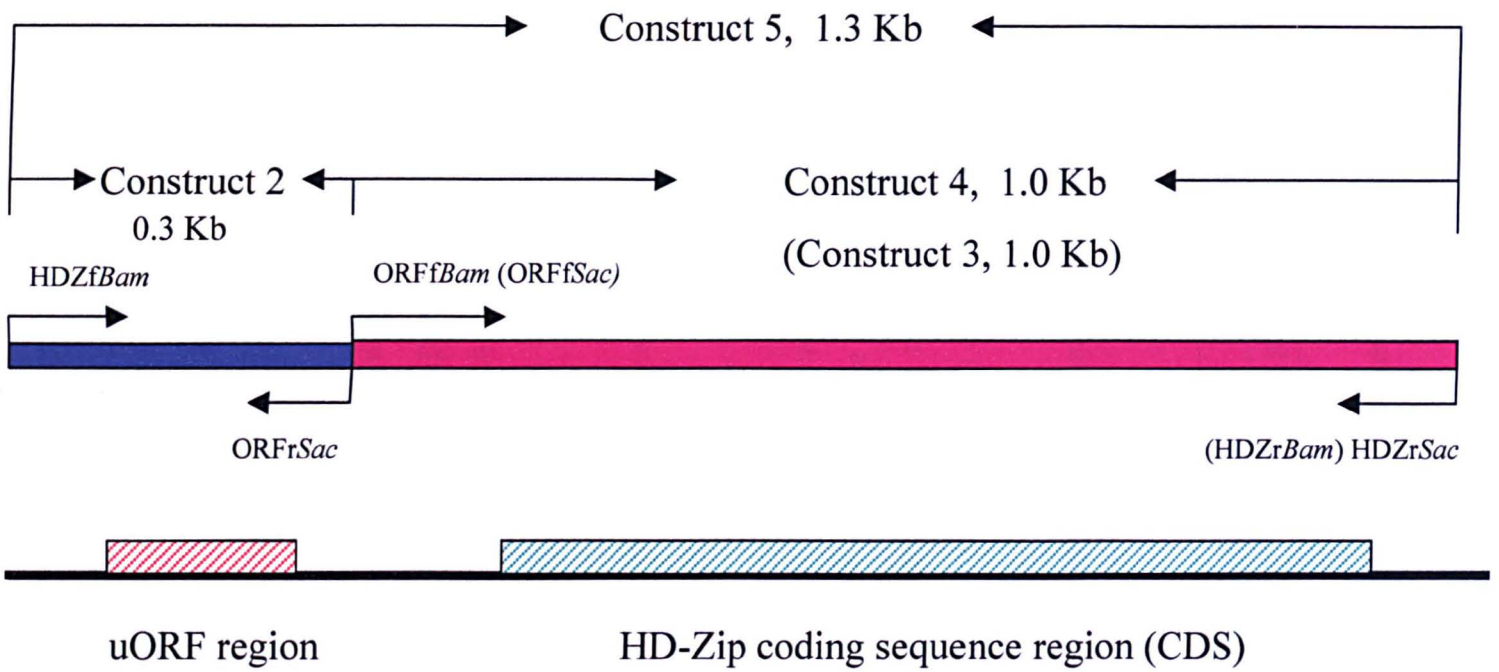


Figure 4.2 Schematic map of HD-Zip gene fragment. Construct 2, sense uORF; Construct 3, antisense HD-Zip; Construct 4, sense HD-Zip (CDS); Construct 5, sense full length HD-Zip (uORF +CDS). HDZf*Bam*, ORFf*Bam* , ORFf*Sac*, ORFr*Sac*, HDZr*Bam*, HDZr*Sac* are primers used for amplifying the fragments.

Table 4.2: Fragment length of the insert and primers used to amplify the insert HD-Zip fragments.

Fragment number	PCR fragment for insert	Primer used	Size (Kb)
1	Sense HD-Zip full length (CDS + uORF)	$HDZf_{Bam}$ + $HDZr_{Sac}$	1.3
2	Sense HD-Zip coding sequence (CDS)	$ORFf_{Bam}$ + $HDZr_{Sac}$	1.0
3	Antisense HD-Zip coding sequence	$ORFf_{Sac}$ + $HDZr_{Bam}$	1.0
4	Sense uORF (uORF)	$HDZf_{Bam}$ + $ORFr_{Sac}$	0.3

4.2.2.2 Purification of DNA fragments obtained from PCR by GeneClean III Kit

The PCR reaction products produced above (10 μL from each sample of fragment 1, 2, 3 and 4) were subjected to electrophoresis on a 1% or 1.2% (w/v) agarose gel and stained with ethidium bromide. The band from each sample was cut out and was processed through the GeneClean procedure following the manufacturer's instructions (name of the product supplier).

4.2.3 Ligation of HD-Zip gene fragments with pGEM-T easy vector

The purified HD-Zip gene fragments were ligated into the pGEM-T Easy vector. Each ligation was set up in a volume of 10 μL, including 5 μL 2X rapid ligation buffer, 1 μL pGEM-T easy vector, 1 μL GeneClean Kit IIIpurified PCR products, 1 μL ligase and 2 μL H_2O. The ligation was incubated at 4°C overnight.

4.2.4 Transformation of HD-Zip gene fragments and pGEM-T into XL 10-Gold Kan ultracompetent cells

The ligations of HD-Zip gene fragments with pGEM-T easy vector were transformed with competent *E. coli* strain XL-10 cells. The detailed procedures of the transformation were carried out following the instruction provided by the kit supplier (Stratagene). Four pure white single colonies from each transformation were chosen and inoculated into a

tube containing LB-ampicillin liquid medium, respectively. The cultures were grown at 37°C with shaking overnight.

4.2.5. The cloned fragments checked by double digestion, PCR and sequencing

PCR reactions and double digestion were carried out to confirm the transformations. The plasmid DNA samples isolated from the transformants were used as templates for PCR with appropriate pairs of primers. The details of the PCR reactions are as described in **4.2.2.1**. The double digestion was done as described in **4.2.1.4**.
Twoμg plasmid DNA sample containing the relevant insert (confirmed by PCR and double digestion assays) was prepared using Qiagen plasmid DNA Minipreps purification Kit for sequencing.

4.2.6 Isolation of HD-Zip gene fragments for vector construction

Plasmid DNA samples carrying inserts confirmed to have the correct sequence were digested with *Bam*HI and *Sac*I to cut out the HD-Zip gene fragment inserts. The double digestion products were analysed by agarose gel electrophoresis. The bands of interest containing the inserts were cut out of the gel and the Geneclean procedure was used to generate high quality insert DNA samples for vector construction.

4.2.7 Ligation of pBECKS400/6 and the inserts

The GeneClean purified HD-Zip gene fragments from **4.2.6** as inserts were ligated with Geneclean purified pBECKS400/6 vector. Each ligation was set up in a volume of 10 μL including 5 μL 2X rapid ligation buffer, 1 μL pBECKS400/6 vector, 2 μL of the insert, 1 μL ligase and 1 μL H_2O. The ligation was incubated at 4°C overnight.

4.2.8 Transformation of PA-3 pBECKS400/6 vector constructs into XL10-Gold cells

Each ligation from the last step was used to performed transformation with XL10-Gold Kan Ultracompetent Cells. The detailed procedures were carried out as described previously.

4.2.9 Transformation of the constructs with *Agrobacterium tumefaciens* by electroporation

4.2.9.1 Preparation of competent *A. tumefaciens* cells

A. tumefaciens LBA 4404, taken from a fresh streak plate, was inoculated into 20 mL of LB medium held in a 50 mL sterile screw-capped centrifuge tube. The suspension culture was incubated for 2 days at 28°C in an orbital incubator (170 rpm) with the tube held horizontally to ensure full air/medium mixing. Between approximately 16 and 40 hours of incubation, the tube was unsealed under sterile conditions for 20-30 seconds and the contents allowed to equilibrate with the air (with slight occasional 'swirling' of the culture). The tube was then resealed and returned to the incubator for a further 4 hours to allow the recovery of high numbers of viable cells. The A_{600} reading was allowed to increase from approximate 0.5 to 0.8 during this second incubation period. Cells were chilled on ice for 5 min and harvested by centrifugation at 3,000 rpm and then washed three times with ice-cold 10% (v/v) glycerol. The final pellet was re-suspended in 200 µL 10 % (v/v) glycerol and aliquots of 40 µL were used immediately or stored at minus 70°C.

4.2.9.2 Preparation of Plasmid

Standard techniques were used to grow and manipulate all pBECKS400/6 constructs containing relevant insert in *E. coli* XL10 strains. Plasmid DNA was isolated using Qiagen Plasmid DNA Isolation Minipreps Kit.

4.2.9.3 Electroporation

Forty μL of the competent cells were mixed with approximately one μg plasmid DNA (<10 μL volume) and electroporated in a 0.1 cm cuvette (ice-chilled) using a BIO-RAD Gene Pulser set to parameters 12.5 Kv/cm, 25 μF, 400 hms, timepulse 4-8 msec. Cells were diluted with 1 mL LB medium and grown for 2 hours at 28°C in an orbital incubator. One hundred μL of suspension culture was plated onto a LB-agar plate containing spectinomycin (100 mg L^{-1}) in duplicate. The plates were incubated at 28°C for 2-3 days.

4.2.9.4 Confirmation of the electroporated transformation

Small scale plasmid preparation from transformed, spectinomycin- resistance *Agrobacterium* colonies was processed by alkaline lysis, as for *E. coli* plasmid. This could therefore be used to confirm the presence of the introduced binary plasmid and further PCR assay using such plasmid as template or double restriction digestion to check against recombination events.

4.2.10 Analysis of HD-Zip gene sequence by BLAST homology searches

The consensus sequence of HD-Zip full length gene was used to search for homologous sequences using either the TBLASTX protocol directly, or the BLASTP protocol with the translated protein sequence.

4.2.11 Plant materials

An autotetroploid line of alfalfa (*Medicago falcata*) 47/1-5, which had been selected for its high capacity for direct somatic embryogenesis (Denchev *et al.*, 1990) was used. Sterile plants were maintained on MS medium under the condition as described in section **2.2.1.**

4.2.12 *A. tumefaciens* strain and constructs used for the transformation

Agrobacterium tumefaciens strain LBA4404 were utilised as transforming bacteria. The vector used in this chapter was pBECKS400/6 (McCormac *et al.*, 1997) under the control

of the CaMV35S promoter, carrying the *nptII* selectable marker conferring kanamycin resistance to transformed plant cells. The vector constructs were established and introduced into *A. tumefaciens* LBA4404 *via* electroporation as described in Section **4.2.9.3**. The strains used for sense and antisense transformation of alfalfa plants in this chapter were LBA4404·PA-3 containing: A2-1-1-2 (sense uORF insert, construct 2), D3-1-1-4 (antisense HD-Zip gene insert, construct 3), F4-6-3-1 (sense HD-Zip gene insert, construct 4) and C5-4-2-1 (sense full-length HD-Zip gene insert, construct 5). They were subcultured in LB-spectinomycin medium and grown as described in **2.2.2.**

4.2.13 *Agrobacterium*-mediated transformation and plant regeneration

The details were carried out as described in **2.2.3.** 12 days induction period on MSH medium was applied to transformation with Construct 4 sense HD-Zip CDS and Construct 5 sense full length HD-Zip.

4.2.14 Re-callusing assay to confirm transformation

Alfalfa leaves from regenerated mature putative transgenic plants of all constructs were chopped and cut into 3-5 mm squares or pieces in liquid MS medium. The plant fragments were placed on solid B_5h (see appendix) medium containing 50 mg L^{-1} kanamycin. Duplication was carried out with each strain. Calli were allowed to develop for 4 weeks, after which growth was evaluated. Control assays were also conducted using untransformed line 47/1-5.

4.2.15 Molecular analysis of transformed plants by PCR

For PCR detection, genomic DNA from transgenic plants was isolated using a Puregene DNA isolation kit (Flowgen) following the manufacturer's instructions. About 100 mg leaf tissue was used for each Miniprep. DNA was recovered by incubation in 100 µL of DNA hydration solution at room temperature overnight to achieve better quality and high yield. PCR was performed in 50 µL reaction mixture consisting of 1x PCR buffer, the four

different deoxyribonucleotides (200 μM each), the two relevant primers (1μL each), 0.5 units of *Taq* DNA polymerase and 2 μL of DNA extract described above. The mixture was heated for 3 min at 94°C, followed by 39 cycles of 94°C for 1 min, 65°C for 1 min, 72°C for 50 sec (for plants transformed with the uORF construct) or 2 min (for the other three constructs), and the final extension was at 72°C for 6 min. An aliquot (15 μL) of the reaction mixture was used for analysis of the amplified products by agarose gel electrophoresis. The primer pairs 5'-ATGACGCACAATCCCACTATCCTT-3' (nucleotides sequence from the CaMV35S promoter) and 5'-CATCGCAAGACCGGCAACAGGATTC-3' (nucleotides sequence from *nos* gene) were used to amplify the insert in each transgenic plant and the control.

4.2.16 Direct somatic embryogenesis of sense and antisense transgenic plants

A direct somatic embryogenesis system (Denchev, 1991a) with relevant modifications was used. The basic procedures are the same as described in chapter 2 and 3, but variable 2,4-D induction periods were tested.

Young trifoliate leaves (500 mg fresh weight) from 8-12 day-old 47/1-5 sense or antisense transgenic plants were chopped with a scalpel into small pieces in liquid $B_5$0 medium and then the leaf explants were transferred into 100 mL flasks containing 50 mL of B_5IV liquid induction medium ($B_5$0 supplemented with 4 mg L^{-1} 2,4-D, 0.2 mg L^{-1} kinetin, 1 mg L^{-1} adenine and 10 mg L^{-1} glutathione. See Appendix). The cultures were maintained on rotary shaker at 100 rpm under a 16/8-hour photoperiod at 22°C. The induced suspension cultures were transferred to embryo development medium $B_5$3M (2,4-D-free medium) on days 1, 10, 18 and 26. Cultures induced for one day were subsequently incubated for 17-days in B_5IV medium without 2,4-D before transferring to $B_5$3M medium. Untransformed line 47/1-5 and transgenic line *crk1-17-1-2* derived from 47/1-5 (Chapter 2) were used as controls.

The transgenic plants with each individual construct of four were used as A2-1-1-1 (uORF), D3-1-1-1 (antisense HD-Zip gene), F4-6-4-2 (sense HD-Zip gene) and C5-4-1-5 sense full length HD-Zip gene. Four flasks of the suspension culture were set up for the transgenic plant with each construct. After 60 days in suspension culture, the number of

the embryos formed from the plants with all the four constructs were counted, respectively.

4.3 Results

4.3.1 Sequence analysis of HD-Zip gene

Sequence analysis by TBLASTP homology searching has shown that this alfalfa HD-Zip gene shares sequence homology with many HD-Zip genes in *Arabidopsis*, soybean, tomato, rice, carrot, etc., The gene shows closest homology to the *Phaseolus vulgaris* homeodomain gene, but of the *Athb* gene family in *Arabidopsis,* the HD-Zip shows greatest similarity to ATHB-1 HD-Zip transcription factor. One of the most striking features of the sequence comparison with *Athb*-1 is that apart from the similarity of the two HD-Zip protein sequences, there is a near-perfect match of the uORF sequence, which is not found in any other members of the large Athb gene family.

The submitted nucleotide sequence and protein sequence of HD-Zip gene were given below:

Nucleotide sequence:

* * 10 * 20 * 30 * 40 * 50 * 60

GGCTCCCTCTCTCTCGCACGCACACACCAAAAAACCTCGCCGGGAAAACTCCATCTCCGG
CCACCGGCGTTCTCCTTCCTCCGGTAAACCCTTCAGTTCAAAACTGAATCCCTTCAAAGA
CCACCGGAATCGGTGGTTTTTACCTTACAGTGCATGATGGGATCTTGCATATGTCCGTTA
GAAACTCCAGCAAGGTTGCTTTGGACAACAAGTTTCTTCCGTCATAAGTTAATGATCTTT
TAATCCACAAATCATCATAGAAATATTTCTATAAAAAAAATATCAAGTTTGGAATTAGAA
AGAAAAAATATATATATCATTTTTTGTTTCTGATTCTGAAAGATTAATAGTATTAAGAGA
AAATTTGTAGCACAACCTTTAACGTGAAGTTTTGAAGTTTGAAGTTGAAAAAATGGAGTC
TGGTCGTCTTTACTTTGATTCTCCTGCTTGTCGTGGGAATAACATGAACATGCTGTTTCT
TGGAAATGCTGATCTTGGTTTTCGAGGAAGGTCAATGATGAGCATGGGGGAAGGCTCAAA
AAGGAGACCTTTCTTTAGCTCACCGGATGAACTGTATGATGAGGAGTACTACGAAGAACA
GTCACCGGAGAAGAAGCGCCGCCTCACTTCCGAGCAGGTCCATATGTTGGAGAAGAGCTT

TGAGGAAGAGAACAAACTTGAGCCAGAGAGGAAAACCCAGTTGGCCAAGAAACTAGGATT
GCAACCTAGGCAGGTGGCTGTATGGTTTCAGAACCGTAGGGCTAGATGGAAGACAAAACA
ACTTGAAAGAGATTATGATGTTCTCAAGTCTTCCTATGATTCCCTACTTTCAACATATGA
TTCCATTACTAAGGAGAATGAGAAACTCAAATCTGAGGTGGTATCCTTAAATGAGAAGCT
TCAAGTTCAAGCTAAAGATATGCTTGAAGAACCTTTGTCAGAAAAGAAAGCTGATCCACT
TCCAGTGGACATAGCTCAGATTTTCAGCATCAGGGTGGATGACCACATGAGTTCTGGAAG
CGTTGGAAGTGCAGTGGTGGACGAGAGTAGCCCCCGTGTCGTTGGTGTCATTGTTGACAG
CGTCGATTCATACTTTCCAGCTGACAACTATGCTGGATGTGTGGCTCCTATTGAAAGAGT
TCAATCAGAGGAAGATGATGGGAGTGATGGGAGGAACTACTTTGATGTGTTTGTTGCATC
TGAAACTGAGCAACATAACCATGAAGAGGGAGAGGCACTGAATTGGTGGGGTAATATGTA
TTATGTTGCATAATCAAGATCAAGAACATTGTATAATTAGATACATCATGTAATTTGGGT
GATTTGGGCGCCTAGGTGTTGAAATGTCTTTAAGCCAAGCTTAGTAAAATAAAAAGTCCA
GTAATTTGGGCAATGGCGTCCTTTGTTGTACTTAAAAAAAATTTATAATAAACTATTGAA
GTTAAGCAAAAAAAAAAAAAAAAAAAAAAAAA

Note: uORF (154-242 bp) and HD-Zip coding sequence (412-1272 bp) regions were marked with underline.

Protein sequence:

GSLSLARTHQKTSPGKLHLRPPAFSFLR*TLQFKTESLQRPPESVVFTLQCMMGSCICPL
ETPARLLWTTSFFRHKLMIF*STNHHRNISIKKISSLELERKNIYIIFCF*F*KINSIKR

KFVAQPLT*SFEV*S*KNGVWSSLL*FSCLSWE*HEHAVSWKC*SWFSRKVNDEHGGRLK

KETFL*LTG*TV**GVLRRTVTGEEAPPHFRAGPYVGEEL*GREQT*AREENPVGQETRI
AT*AGGCMVSEP*G*MEDKTT*KRL*CSQVFL*FPTFNI*FHY*GE*ETQI*GGILK*EA

SSSS*RYA*RTFVRKES*STSSGHSSDFQHQGG*PHEFWKRWKCSGGRE*PPCRWCHC*Q

RRFILSS*QLCWMCGSY*KSSIRGR*WE*WEELL*CVCCI*N*AT*P*RGRGTELVG*YV

LCCIIKIKNIV*LDTSCNLGDLGA*VLKCL*AKLSKIKSPVIWAMASFVVLKKNL**TIE
VKQKKKKKKKKX

The key results of WU-TBLASTX of full length HD-Zip gene were shown as following (The details were shown in Appendix):

TBLASTX 2.0MP-WashU [23-May-2003] [decunix5.0a-ev56-IP32LF64 2003-05-23T14:40:20]

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Reference:  Gish, W. (1996-2003) http://blast.wustl.edu

Query=  Sequence
        (1478 letters)

  Translating top strand of query sequence in 3 reading frames

Database:  embl
           3,238,123 sequences; 9,788,945,129 total letters.
Searching....10....20....30....40....50....60....70....80....90....100% done
WARNING:  hspmax=1000 was exceeded by 111 of the database sequences, causing
          the associated cutoff score, S2, to be transiently set as high as
          46.
```

Sequences producing High-scoring Segment Pairs:	Reading Frame	Score	P(N)	N	Smallest Sum High	Probability
EM_PL:AF402606 AF402606.1 Phaseolus vulgaris homeodoma...	+1	941	5.7e-95	2		
EM_PL:ATHB1 X58821.1 A.thaliana homeobox gene Athb-1 mRNA	+1	771	6.3e-88	2		
EM_PL:AF443621 AF443621.1 Craterostigma plantagineum h...	+1	704	4.8e-80	2		
EM_PL:AK119592 AK119592.1 Oryza sativa (japonica culti...	+3	524	1.2e-56	2		
EM_PL:DCKUROC D26575.1 Carrot mRNA for DNA-binding pro...	+1	453	1.2e-40	1		
EM_PL:AF184278 AF184278.1 Glycine max homeodomain-leuc...	+1	411	2.3e-40	2		
EM_PL:ATHOMEOB M90416.1 Arabidopsis thaliana homeobox ...	+1	443	6.7e-39	1		
EM_PL:AF402605 AF402605.1 Phaseolus vulgaris homeodoma...	+1	392	4.6e-34	1		
EM_PL:AF268422 AF268422.1 Brassica rapa subsp. pekinen...	+1	359	4.9e-34	2		
EM_PL:AF402604 AF402604.1 Phaseolus vulgaris homeodoma...	+1	360	6.9e-34	2		
EM_PL:AY101610 AY101610.1 Nicotiana tabacum homeodomai...	+1	356	3.4e-33	2		
EM_PL:AF208044 AF208044.1 Arabidopsis thaliana homeodo...	+2	346	4.1e-33	2		
EM_PL:LEVAHOX1G X94947.1 L.esculentum mRNA for homeobo...	+3	374	6.8e-33	2		
EM_PL:AY202115 AY202115.1 Arabidopsis thaliana sequenc...	-1	376	4.3e-32	1		
EM_PL:AP006364 AP006364.1 Lotus corniculatus var. japo...	-1	373	7.3e-32	3		
EM_PL:AC010870 AC010870.6 Arabidopsis thaliana chromos...	-3	277	1.3e-31	2		
EM_PL:AF443623 AF443623.1 Craterostigma plantagineum h...	+1	368	1.5e-31	1		
EM_PL:AB042766 AB042766.1 Zinnia elegans ZeHB7 mRNA fo...	+1	366	4.6e-31	1		
EM_PL:AF443620 AF443620.1 Craterostigma plantagineum h...	+1	362	6.9e-31	1		
EM_PL:AY063819 AY063819.1 Arabidopsis thaliana putativ...	+3	357	1.8e-30	1		
EM_PL:ATATHB6 X67034.1 A.thaliana mRNA Athb-6	+1	357	1.9e-30	1		

Results of ORF region sequence TBLASTX were shown as following:

```
TBLASTN    2.0MP-WashU    [23-May-2003]    [decunix5.0a-ev56-IP32LF64    2003-05-
23T14:40:20]

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All Rights Reserved.

Reference:  Gish, W. (1996-2003) http://blast.wustl.edu

Query=  Sequence
        (29 letters)

Database:  embl
           3,238,123 sequences; 9,788,945,129 total letters.
Searching....10....20....30....40....50....60....70....80....90....100% done
```

```
WARNING:  hspmax=1000 was exceeded by 1 of the database sequences, causing the
          associated cutoff score, S2, to be transiently set as high as 26.

                                                                    Smallest
                                                                      Sum
                                                  Reading  High   Probability
Sequences producing High-scoring Segment Pairs:     Frame Score   P(N)       N

EM_PL:ATHB1 X58821.1 A.thaliana homeobox gene Athb-1 mRNA +1    145  2.8e-07    1
EM_PL:AF443621 AF443621.1 Craterostigma plantagineum h... +2    140  1.0e-06    1
EM_PL:AK119592 AK119592.1 Oryza sativa (japonica culti... +2     88  0.35       1
```

Multiple view of uORF BLAST results:

```
Identities computed with respect to: (query) Sequence
Maximum sequences to show: 13
Colored by: identity + property

HSP processing: ranked

                                          1 [        .         .         ]
29
    Sequence          1:29                MMGSCICPLETPARLLWTTSFFRHKLMIF
  1 EM_PL:ATH553233   1:29      195:281   MMGFCICPLESPARLLWSTSFFRHKIMIF
  2 EM_PL:ATHB1       1:29      121:207   MMGFCICPLESPARLLWSTSFFRHKIMIF
  3 EM_PL:AY058188    1:29      160:246   MMGFCICPLESPARLLWSTSFFRHKIMIF
  4 EM_PAT:AX510120   1:29     1791:1877  MMGFCICPLESPARLLWSTSFFRHKIMIF
  5 EM_PAT:AX652685   1:29     1791:1877  MMGFCICPLESPARLLWSTSFFRHKIMIF
  6 EM_PL:AC009325    1:29     6235:6149  MMGFCICPLESPARLLWSTSFFRHKIMIF
  7 EM_PL:AF443621    1:29      125:211   MMGFCICPLDTPARLLWCSSFFRHKLMLF
  8 EM_PL:AK119592    1:26      281:358   MMGFSLTPMKISTRLLWSTSFFRHKI---
  9 EM_PAT:AX655116   1:26     1739:1816  MMGFSLTPMKISTRLLWSTSFFRHKI---
 10 EM_PL:AP004888    1:26  128681:128604 MMGFSLTPMKISTRLLWSTSFFRHKI---
 11 EM_PL:AK101569    2:26      259:333   -MGFSLYPMKTSTRMLWSTSFFRHKV---
 12 EM_PL:AK100634    2:26      259:333   -MGFSLYPMKTSTRMLWSTSFFRHKV---
 13 EM_PL:AP004667    2:26    49035:48961 -MGFSLYPMKTSTRMLWSTSFFRHKV---
```

4.3.2 Generation of the donor vector pBECKS400/6

In order to generate the donor vector pBECKS400/6, *E. coli* competent strain XL10 cells were transformed with the plasmid. The plasmid DNA samples derived from four selected single colonies PA-1, PA-2, PA-3 and PA-4 were examined by *BamH*I and *Sac*I single and double restriction digestion. All four strains showed the same restriction digestion patterns (Figure 4.3). PA-3 was chosen to be used in the construction. The band of *BamH*I and *Sac*I double digestion products separated by gel electrophoresis were cut and processed using the GeneClean procedure. The GeneClean products containing opening

vector pBECKS400/6 with *BamH*I and *Sac*I two restriction ends were ready for use as donor vector.

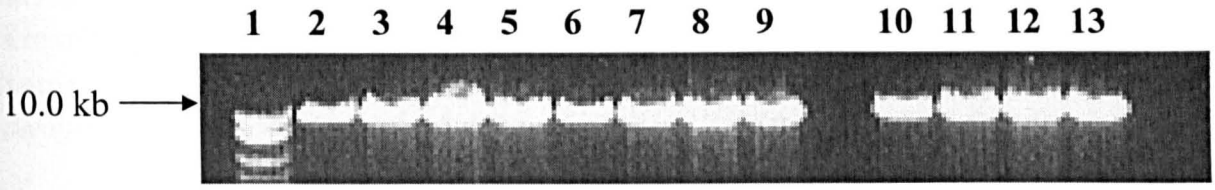

Figure.4.3 Gel electrophoresis photo to show restriction digestion products of plasmid pBECKS400/6 by *BamH*I (B), *Sac*I (S) single digestion and B+S double digestion. Lane 1, marker; lane 2-5, *BamH*I single digestion; lane 6-9, *Sac*I single digestion; lane 10-13, B+S double digestion.

4.3.3 Generation of the full-length HD-Zip gene template $Y_{2\text{-}5}$

To generate the full-length HD-Zip gene template, several techniques were involved. First, the core sequence of the HD-Zip gene isolated by subtractive cloning technique was used to design the primers for 5'-RACE and 3'-RACE PCR to generate the full-length HD-Zip gene sequence. Then the complete sequence was used to design primers for PCR to isolate the full-length HD-Zip gene fragment using cDNA as a template. The cDNA was synthesized by reverse transcription of total RNA. The full-length HD-Zip gene sequence isolated by PCR was subcloned into pGEM-T vector. After restriction digestion and sequence analysis, $Y_{2\text{-}5}$ was chosen to be used as the template to isolate uORF, HD-Zip and full-length HD-Zip fragments. The sequence of $Y_{2\text{-}5}$ is shown in Fig.4.4

TNNACCNCCAGCTTTTAGGTGACACTATAGAATACTCAAGCTATGCATCCAACGCGTTGGGAGCTCTCCC
ATATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGATTCTGCAGCCTCCGGTAAACCCTTCAGTTCA
AAACTGAATCCCTTCAAAGACCACCGGAATCGGTGGTTTTTACCTTACAGTGCATGATGGGATCTTGCAT
ATGTCCGTTAGAAACTCCAGCAAGGTTGCTTTGGACAACAAGTTTCTTCCGTCATAAGTTAATGATCTTT
TAATCCACAAACCATCATAGAAATATTTCTATAAAAAAAATATCAAGTTTGGAATTAGAAAGAAAAAATA
TATATATCATTTTTTGTTTCTGATTCTGAAAGATTAATAGTATTAAGAGAAAATTTGTAGCACAACCTTT

AACGTGAAGTTTTGAAGTTTGAAGTTGAAAAAATGGAGTCTGGTCGTCTTTACTTTGATTCTCCTGCTTG
TCGTGGGAATAACATGAACATGCTGTTTCTTGGAAATGCTGATCTTGGTTTTCGAGCAGGAAGGTCAATG
ATGAGCATGGGGGAAGGCTCAAAAAGGAGACCTTTCTTTAGCTCACCGGATGAACTGTATGATGAGGAGT
ACTACGAAGAACAGTCACCGGAGAAGAAGCGCCGCCTCACTTCCGGCAGGTCCATATGTTGGAGAAGAGC
TTTGAGAAGAGAACAACTTGGCCAGAGAGGAAAACCCAGTGGCAAGAACTAGATGCACCTAGCAGTGGTG
ATGGTTCAGACCGAGGCTGATGGAACAAACACTTGAGAATATGAGNTCTCAGCTTCTAGATCCTCTTCAC
ANTGATCNTACTAGAGAGAGACTAATTGGGGGATCTAANAAACTCAGTCAGCTAGATTNTGAGACTTGCA
GAANAGTGTCNTTCGNGCTANTAATTCACTNGGGNTACCT

Figure 4.4 The nucleotide sequence of full length HD-Zip gene in $Y_{2\text{-}5}$, including uORF and HD-Zip coding sequence regions.

4.3.4 Generation of the HD-Zip gene fragment inserts

$Y_{2\text{-}5}$ was used as a template for PCR with primer pairs containing *BamH*I and *Sac*I cohesive ends, as shown in **4.2.2.1**. Sense uORF, sense and antisense HD-Zip CDS and sense full-length HD-Zip gene fragments were generated. These fragments were subcloned into pGEM-T and checked by DNA sequencing. The selected strains A2-1, D3-1, F4-6 and C5-4 containing relevant inserts of sense uORF, antisense HD-Zip CDS, sense HD-Zip CDS and sense full-length HD-Zip gene respectively were chosen to be used for construct preparation.

4.3.5 Transformation of pBECKS400/6 carrying the inserts with XL10

The purified donor vector pBECKS400/6 (PA-3) and purified inserts of sense uORF, antisense HD-Zip gene, sense HD-Zip gene and sense full-length HD-Zip gene were ligated and transformed into XL10. Four single colonies from each transformation were selected: XL10·PA-3 & 2-1-1~4 (sense uORF insert), XL10·PA-3 & D3-1-1~4 (antisense HD-Zip gene insert), XL10·PA-3 & F4-6-1~4 (sense HD-Zip gene insert) and XL10·PA-3 & C5-4-1~4 (sense full-length HD-Zip gene insert).

4.3.6 Confirmation of the transformation with XL10

In order to confirm the successful transformation of the constructs with XL10, plasmid DNA was isolated from each strain carrying relevant insert and examined by PCR and double restriction digestion assays. Apart from the strain of XL10 PA-3 & A2-1-3, all the strains of the four constructs with XL10 were found to generate bands with the expected size of the original insert by both PCR and double restriction digestion assays. The results were shown on the table 4.3.

Table 4.3 PCR and double restriction assays to check the constructs with XL10

Assay to the insert	XL10·PA-3& A2-1 (sense uORF insert)				XL10·PA-3& D3-1 Strain (antisense HD-Zip gene insert				XL10 PA-3 & F4-6 Strain (sense HD-Zip insert)				XL10·PA-3 &C5-4 Strain (sense full-HD-Zip gene insert)			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
PCR	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Restriction	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+

4.3.7 Introduction of the constructs into LBA4404 by electroporation

The strains XL10· PA-3 & A2-1-1, XL10 PA-3 & D3-1-1, XL10· PA-3 & F4-6-3 and XL10·PA-3 & C5-4-2 were chosen for isolation of plasmid constructs to be introduced into *Agrobacterium* strain LBA4404 by electroporation. Four single colonies from each electroporated transformation were selected for further confirmation. They are LBA4404·PA-3 & 2-1-1-1~4 (carrying the uORF insert), LBA4404·PA-3 & D3-1-1-1~4 (carrying the antisense HD-Zip gene insert), LBA4404·PA-3 & F4-6-3-1~4 (carrying the sense HD-Zip gene insert), LBA4404·PA-3 & C5-4-2-1~4 (carrying the sense full-length HD-Zip gene insert). The selected LBA4404 strains were checked by PCR assay to confirm the presence of the insert. Except for LBA4404·PA-3 & D3-1-1-1, all of the remaining selected LBA4404 strains were successful in the PCR assay, giving a band of a size

consistent with the corresponding original insert. The PCR assay results are shown on Table 4.4.

Table 4.4 PCR assay to check the constructs with LBA4404

Assay to the insert	LBA4404 PA-3 & A2-1-1 Strain uORF insert)				LBA4404·PA-3& D3-1-1 Strain (antisense HD-Zip insert)				LBA4404 PA-3 & F4-6-3 Strain (sense HD-Zip insert)				LBA4404 PA-3 & C5-4-2 Strain (sense full-length HD-Zip gene insert)			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
PCR	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+

4.3.8 Generation of transformants and formation of fused cotyledons from the normal MSH system

The standard MSH system (Shao *et al.*, 2000; Atanassov *et al.*, 1998) based on direct somatic embryogenesis was used to generate transgenic plantlets via *Agrobacterium*-mediated transformation. In this system, the growth regulator combination during a 10-day induction period was 22.6 μM 2,4-D and 4.7 μM kinetin. Under these conditions, leaf explants were highly embryogenic and only a small number of calli were formed. In the transformation with all four constructs, small globular somatic embryos were observed after 10 days incubation on MS medium and a "wave" of somatic embryogenesis on the surface of the explants occurred during the subsequent 10 days of incubation. Relatively little callus proliferation occurred and after a further period in culture, bottle-shaped or cotyledonary somatic embryos were formed on some explants. During this period, a significant variation in the pattern of regeneration was found between cultures transformed with different constructs. In the sense uORF construct, bottle-shaped somatic embryos occurred within a total of three weeks and whole plantlets were formed in ten weeks. In the other three constructs, the progress of regeneration was found to be much slower, and more difficult.

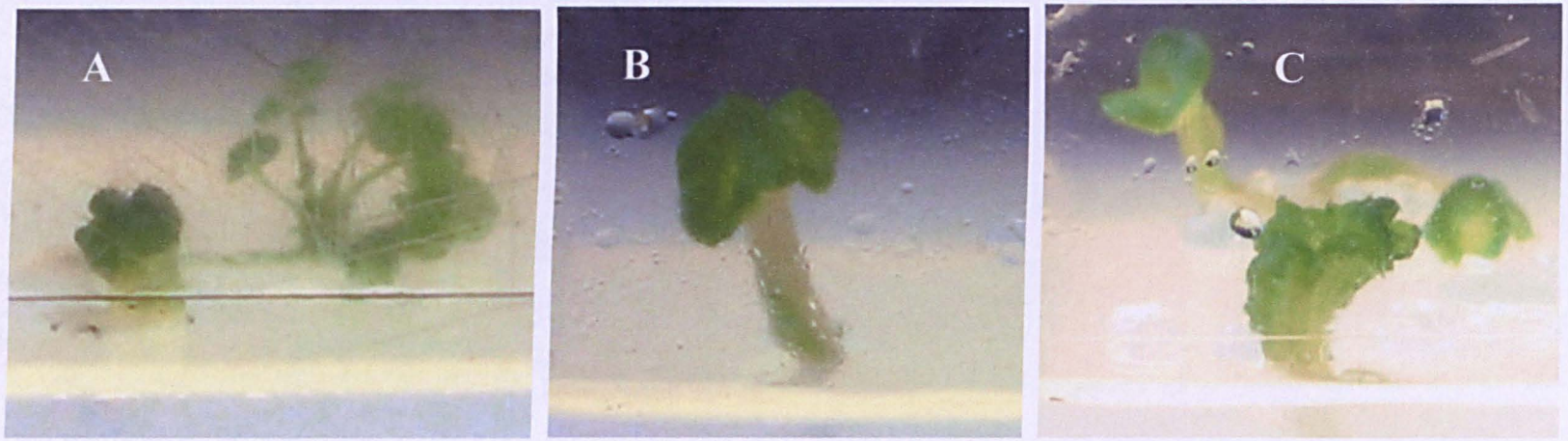

Figure 4.5 Fused single cotyledons were produced from transformed plants with construct 4 (sense HD-Zip) (B, F4-6) and Construct 5 (sense full length HD-Zip) (C, C5-4) by the standard MSH regeneration system. A, antisense HD-Zip transformed plant (D3-1).

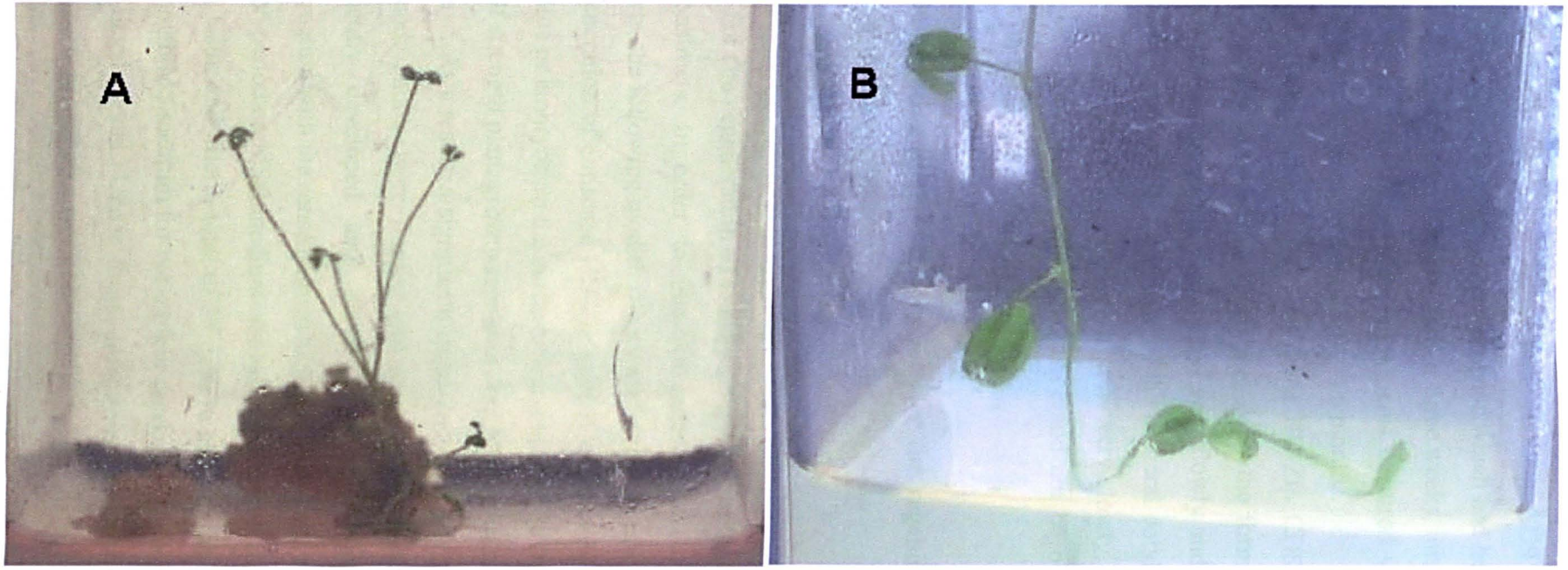

Figure 4.6 Plantlets with a normal shape were generated from transformed explants with Construct 4 and Construct 5 via the modified MSH system. A, sense HD-Zip transgenic plant; B, sense full length HD-Zip transgenic plant.

In the transformation with the antisene HD-Zip CDS construct, direct and indirect embryos were first observed on leaf explants which had been a total of 38 days in culture. The first shoots appeared within a further three weeks, but the subsequent development of these shoots to trifoliolate leaves with stems occurred very slowly. The generation of whole plants therefore took between 4½ to 5 months.

In contrast, transformation with the sense HD-Zip CDS and full length HD-Zip gene constructs produced a unique phenotype. After the formation of bottle-shaped somatic embryos, further development proceeded very slowly and formed only cup-shaped fused cotyledons (Figure 4.5 B, C) instead of the bilateral cotyledon normally produced in the MSH system. These fused cotyledons grew very slowly, then stopped growing, became brown and finally died. Whole plantlets could not be obtained from the normal MSH system with these two constructs.

4.3.9 Modification of the MSH system for the regeneration of sense and antisense HD-Zip transgenic plants

The observations described in section **4.3.8** indicated that the normal MSH system was not suitable for the regeneration of transgenic plants containing sense or antisense HD-Zip gene constructs. In order to improve regeneration of transgenic plants with these constructs, the following modification were integrated into the MSH system:

For regeneration of antisense HD-Zip gene (Construct 3) transgenic plants, leaf explants with callus or embryos on the surface after a total of 28 days in culture were sub-cultured onto half strength plant growth regulator-free MS medium with antibiotics for a further 15 to 20 days. As a result, a significant improvement in the growth of the calli and embryos was found. Shoots were formed and recovered to produce trifoliolate leaves. Stems subsequently developed and whole plants were generated by 3½ months. In transformation with the sense constructs (Construct 4 and Construct 5), the normal ten-day induction period on MSH medium was extended to twelve days. The leaf explants became swollen faster and after a total of 30 days in culture, the explants were subcultured onto half strength MS medium for two to four weeks and then transferred onto MS medium for further development. Under these conditions, bottle-shaped or cotyledonary somatic

embryos developed into shoots and were then recovered to form trifoliolate leaves with stems. It took 4½ to 6 months to regenerate normal whole plantlets carrying these transgenes.

Alternatively, an overnight treatment with B_5IV liquid medium (containing 2,4-D) of callus produced from explants transformed with these two constructs was found to improve the efficiency of regeneration. An integration of a period of subculture on half strength MS medium into the MSH regeneration procedure has been found to shorten the period of the formation of the transgenic plantlets. In addition, no cup-shaped fused cotyledons were produced in these modified MSH systems (Figure 4.6).

4.3.10 Confirmation of transgenic plants by re-callusing

In order to detect the stable integration of transgenes, a recallusing assay with four constructs (sense uORF, antisense HD-ZipCDS, sense HD-ZipCDS and sense full length HD-Zip gene) was conducted. After 33 days incubation on B_5h medium containing kanamycin, callus was generated from all 41 tested plants, except untransformed control plant 47/1-5 (Figure 4.7).

4.3.11 Molecular analysis of transgenic plants by PCR

The plants selected from those transformed with four different constructs were further tested by polymerase chain reaction (PCR) using primer pairs corresponding to the sequence of the CaMV35S promoter and *nos* terminator which flank the regions containing the inserts in the four constructs. These primers amplified a band corresponding in size to the expected insert fragment (~300 bp, 1,000 bp or 1,300 bp, for sense uORF, antisense and sense HD-ZipCDS and full length HD-Zip gene, respectively) plus a vector sequence of 200~300 bp (Figure 4.8A, B, C and D). Positive results were found in most tested but not in non-transformed control plants 47/15 plants (Figure 4.8 A, B, C and D). In Figure 4.8 D Construct 5, PCR assay was carried out two different times (see Figure 4.8 D, a and b). No band was presented in Lane 7 first time (a), but present in second batch of PCR assay (b).

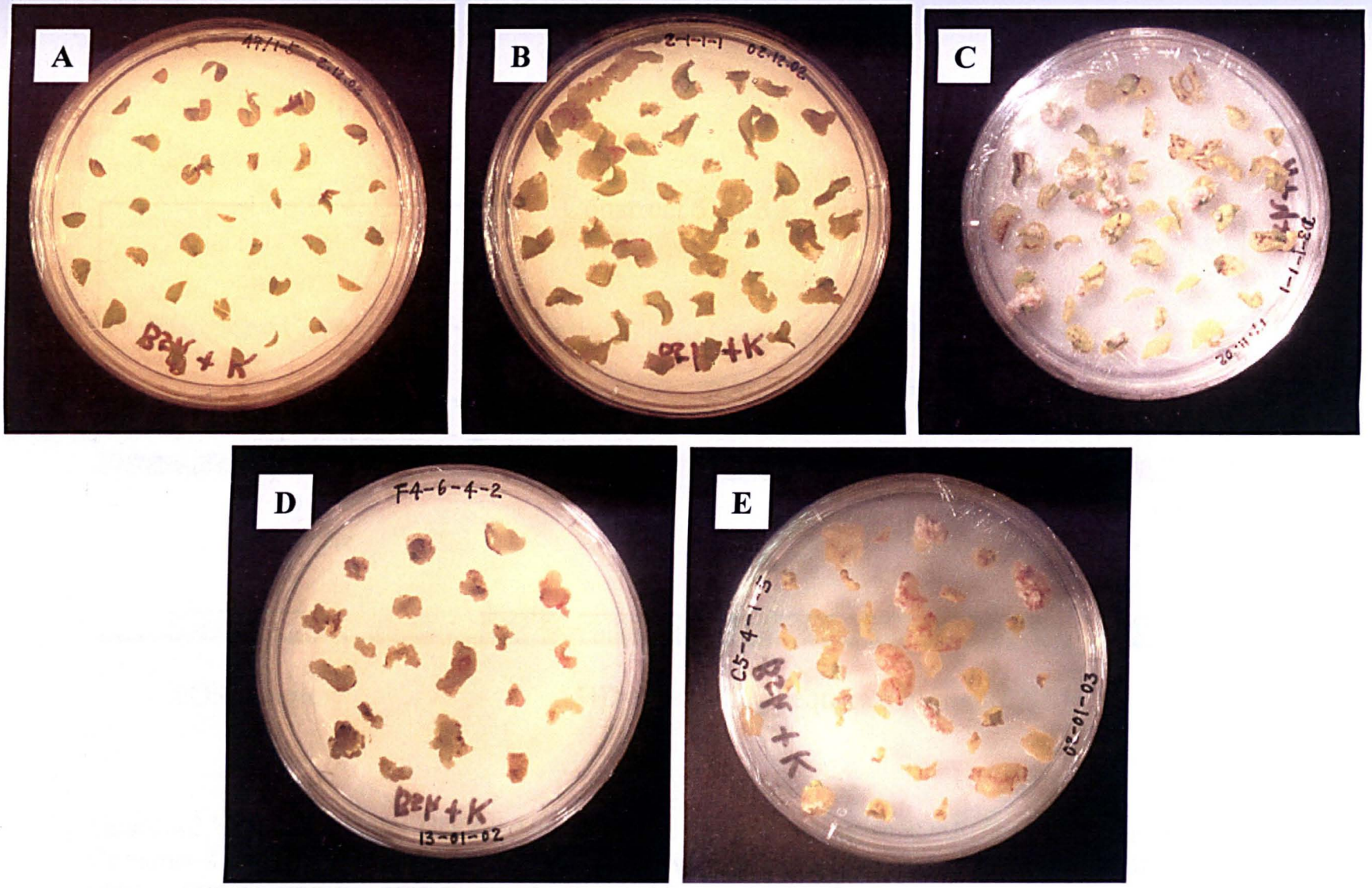

Figure 4.7 Re-callusing assay of explants from regenerated plants transformed with: Construct 2 (sense uORF)(B); Construct 3 (antisense HD-Zip)(C); Construct 4 (sense HD-Zip)(D); Construct 5 (sense full length HD-Zip)(E). A, untransformed plant 47/1-5 (negative control). Calli were grown from all transformed plants B, C, D and E. No callus was found from untransformed plant 47/1-5 (A).

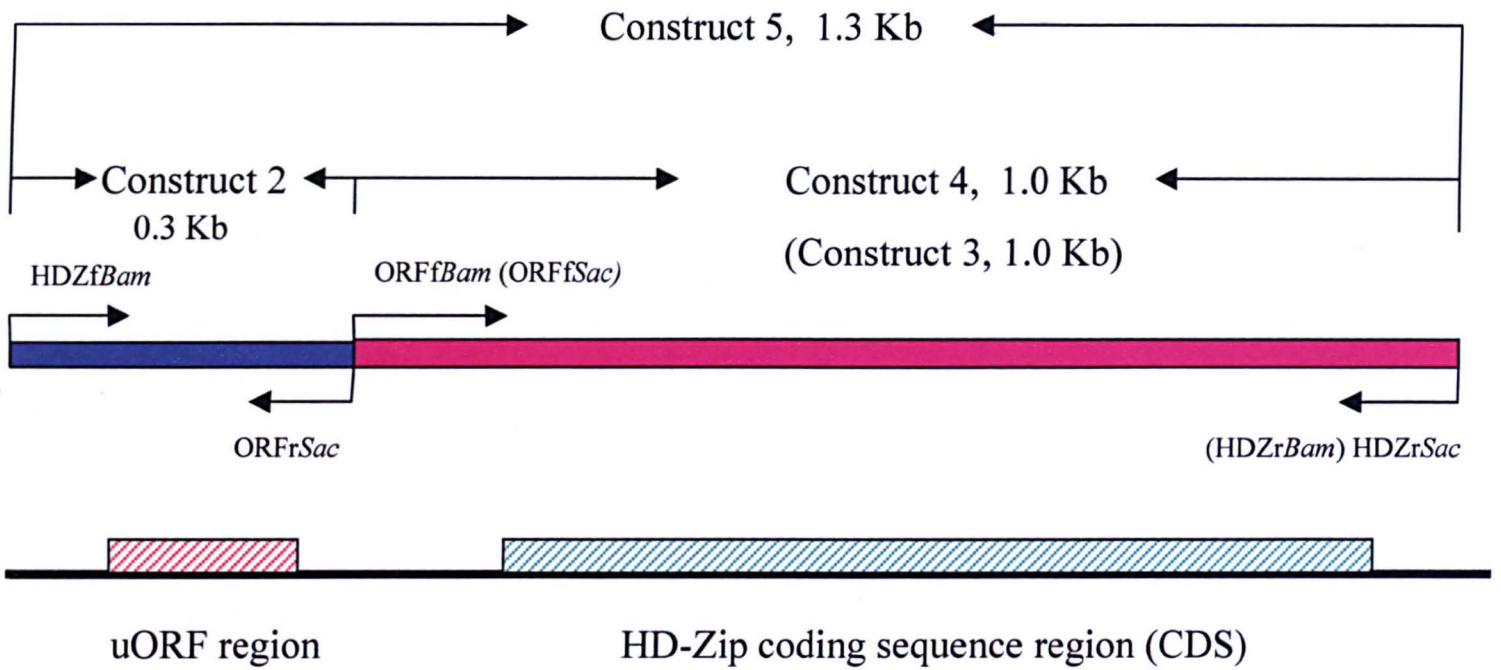

Figure 4.2 Schematic map of HD-Zip gene fragment. Construct 2, sense uORF; Construct 3, antisense HD-Zip; Construct 4, sense HD-Zip (CDS); Construct 5, sense full length HD-Zip (uORF +CDS). HDZf*Bam*, ORFf*Bam* , ORFf*Sac*, ORFr*Sac*, HDZr*Bam*, HDZr*Sac* are primers used for amplifying the fragments.

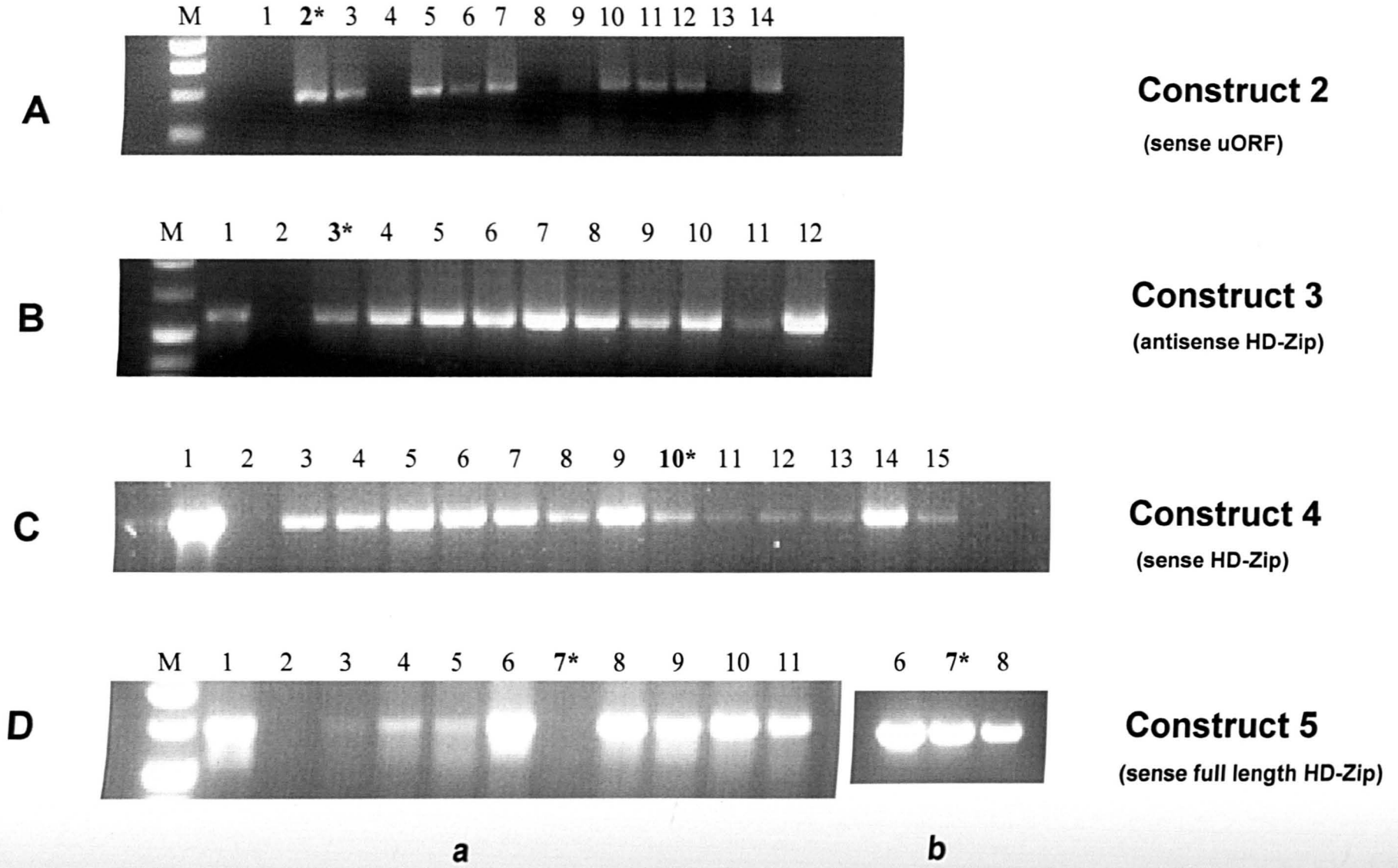
M 1 2* 3 4 5 6 7 8 9 10 11 12 13 14
A
Construct 2
(sense uORF)
M 1 2 3* 4 5 6 7 8 9 10 11 12
B
Construct 3
(antisense HD-Zip)
1 2 3 4 5 6 7 8 9 10* 11 12 13 14 15
C
Construct 4
(sense HD-Zip)
M 1 2 3 4 5 6 7* 8 9 10 11
6 7* 8
D
Construct 5
(sense full length HD-Zip)
a
b

Figure 4.8 PCR analysis of putative transgenic plants carrying different HD-Zip gene fragment constructs.

A, Construct 2 (sense uORF) transgenic plants. M, marker; Lane 1-14, untransformed plant 47/1-5 (negative control), transgenic plant 2-1-1-1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13. No bands was found in Lane 4 (putative transgenic plant 2-1-1-3).

B, Construct 3 (antisense HD-Zip CDS) transgenic plants. M, marker; Lane 1, plasmid of Construct 3; Lane 2, untransformed plant 47/1-5 (negative control); Lane 3-12, putative transgenic plants D3-1-1-1, 2, 3, 3, 4, 5, 6, 7, 8, 9, D3-1-2-1. Bands were found in all transgenic plant, except untransformed plant 47/15.

C, Construct 4 (sense HD-Zip CDS) transgenic plants. Lane 1, plasmid of Construct 4; Lane 2, untransformed plant 47/1-5 (negative control); Lane 3-15, putative transgenic plant F4-6-1-1, 2, 3, F4-6-2-1, 2, F4-6-3-2, F4-6-4-1, 2, 3, 5, F4-6-5-1, 3, 4. Bands were found in all transgenic plant, except untransformed plant 47/15.

D, Construct 5 (sense full length HD-Zip gene) transgenic plants. a) M, marker; Lane 1, plasmid of Construct 5; Lane 2, untransformed plant 47/1-5 (negative control); Lane 3-11, putative transgenic plants C5-4-1-1, 2, 3, 4, 5, 6; b) repeat of samples in Lane 6, 7, 8. Bands were found in all transgenic plant, except untransformed plant 47/15.

*, the transgenic plants were used for the further experiment of somatic embryogenesis.

4.3.12 Comparison of direct somatic embryogenesis between sense and antisense transgenic plants

In order to evaluate the effect of the different transgenes on embryo formation, the transgenic plants were tested using the standard direct somatic embryogenesis system of Denchev *et al* (1991), described in Chapter 2. The number of embryos produced per flask after a total of 60 days suspension culture was calculated (Figure 4.9). The effect of altering the induction period was also determined. After a 10-day induction period, the number of embryos present in all of the samples appeared to be similar. After 18 days induction, Construct 5 (sense HD-Zip gene – i.e. uORF + CDS) transgenic plants showed the highest number of embryos among the evaluated samples and Construct 4 (sense HD-ZipCDS) transgenic plants also produced a much higher number of embryos than those transformed with each of the remaining three constructs. In contrast, after 26 days induction, there was a significant increase in the number of embryos found in both Construct 3 (antisense HD-ZipCDS) transgenic plants and the control plants (*crk1*::*gusA*); whilst there was an obvious decline in the number of embryos observed from Construct 4 and Construct 5 transgenic plants. The experiments with Construct 2 plants showed a slight increase in the number of embryos produced as the induction period was increased from 10 to 26 days.

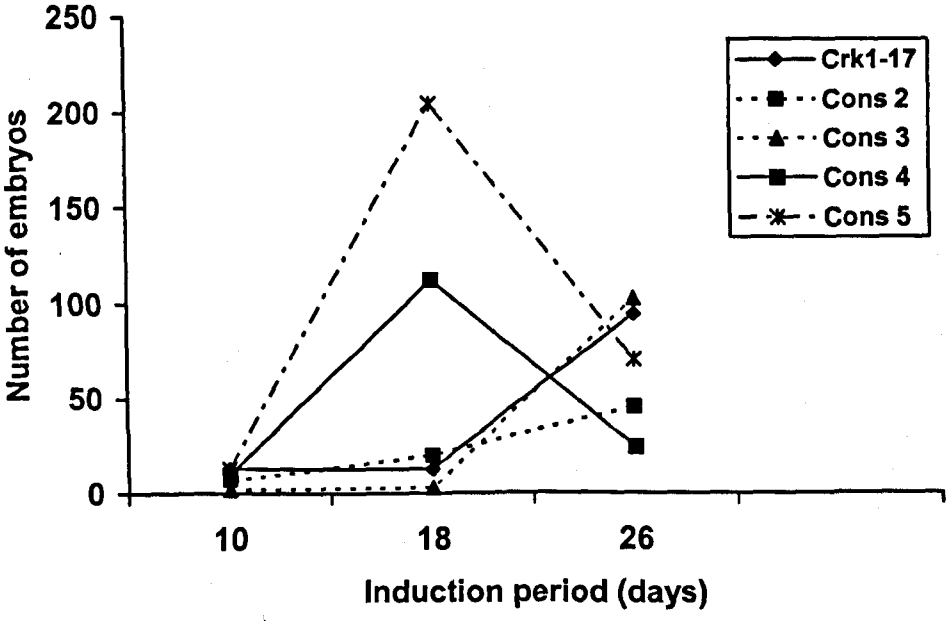

Figure 4.9 Somatic embryogenesis of transgenic plants with four different constructs (Construct 2, 3, 4 and 5) after 60 days subcultured in B53M medium. Crk, constuct *crk1-17-2*; Cons 2, 3, 4 and 5 represent construct 2 (sense uORF), construct 3 (antisense HD-Zip coding sequence), Construct 4 (sense HD-Zip coding sequence) and construct 5 (sense full length HD-Zip), respectively.

4.4 Discussion

4.4.1 pBECKS400/6 is an ideal donor vector for efficient plant transformation

pBECKS400, a member of the pBECKS family of vectors, is a binary T-DNA vector suitable for the introduction of novel genes within established transformation systems. It has been successfully used in the *Agrobacterium*-mediated transformation of several plant species, like carrot, tobacco (Milan, 2002) and alfalfa (Chapter 2). In this study, pBECKS400/6 was chosen as a donor vector for the construction of recombinant plasmids to introduce HD-Zip gene fragments in sense and antisense orientation into

alfalfa plants, due to its several competitive features. First, origins of replication for high plasmid copy number within *E. coli* hosts greatly facilitate the isolation and manipulation of the constructs. The transformed XL10 strains containing a series of HD-Zip gene fragment constructs exhibited a high capacity for the production of high yield plasmid DNA, which satisfied the production of high concentration of plasmid DNA for double restriction digestion, sequencing and electroporation. Secondly, a high frequency of success in producing transformed strains during the process of construction with the insert of interest in this study indicated that pBECKS400/6 is an ideal donor vector for the manipulation of HD-Zip constructs. During the transformation of XL-10, apart from the strain XL10·PA-3 & A2-1-3, all the other 15 selected strains of the four constructs with XL10 were confirmed to be transformed (Table 4.3). Confirmation of the transformation by PCR assay revealed that except for LBA4404·PA-3 & D3-1-1-1, all of the remaining 15 selected LBA4404 strains were successful in transformation with HD-Zip constructs (Table 4.4). These facts demonstrate that all of the confirmed transformed strains were suitable candidates for further vector manipulation or plant transformation. Finally, a chimeric neomycin phosphotransferase (*npt*II) gene for the selection of plant transformants using kanamycin is positioned next to the left border of the T-DNA in order to reduce the possibility of producing truncated sequences of the inserted gene within transformants, thereby providing an effective screen for the transfer of inserted sequences to transformed plants (McCormac *et al.*, 1997). Analysis of HD-Zip transgenic plants by PCR (chapter 7) also demonstrated that the vector used for establishing the constructs in this chapter is efficient in the introduction of the inserts into alfalfa plants.

4.4.2 The importance of the efficient confirmation during generation of the inserts

During the cloning of a gene of interest, it is essential to ensure the fidelity of the insert sequence. To achieve this goal, a strategy for the efficient confirmation of insert fidelity during the cloning procedure was devised. In this study, three techniques: PCR assay, double restriction digestion and DNA sequencing were used for the efficient confirmation of insert fidelity. Thus, PCR and restriction digestion were carried out to check that the insert was the correct length, and that the restriction sites had been correctly maintained.

Only those samples which succeeded in both PCR and double digestion selection were chosen for further sequencing. Furthermore, only those samples which succeeded in all three assays qualified as candidates for the production of the construct insert. Selected samples were used for electroporation to introduce the constructs into *Agrobacterium* strain LBA4404. Analysis of the confirmation of the insert in XL10 and LBA4404 (Table.4.3 and 4.4) showed that, except for one strain in each host (XL10 PA-3 & 2-1-3 and LBA4404·PA-3 & D3-1-1-1), all strains were transformed successfully. These results indicated that the procedures used for establishment of the constructs were generally effective, but not one hundred percent successful. It was therefore necessary to confirm that the *Agrobacterium* strain was transformed with the correct construct before use. In later transformations of alfalfa plants with those constructs, analysis of putative sense and antisense HD-Zip transgenic plants by PCR showed that all of the confirmed transgenic plants included an insert of the correct length. All these results demonstrate that the strategy for the confirmation of insert fidelity by PCR and restriction digestion used in this chapter was efficient and of benefit for the later manipulation of vector construction.

4.4.3 Analysis of the HD-Zip gene *Mfhb-1*

The sequence of the alfalfa HD-Zip gene was generated from the combined sequences of the original HD-Zip clone isolated by subtractive cloning, and the 3' and 5' RACE clones. It includes a 87 bp (from 154 to 242 bp) upstream open reading frame in frame +1 and a 858 bp (from 414 to 1272 bp) HD-Zip coding sequence in frame +2. WU-TBLASTP results of full length sequence revealed that this alfalfa HD-Zip gene shows very high similarity to other HD-Zip genes in some species, such as *Phaseolus vulgaris*, *Arabidopsis*, and rice (see section **4.3.1**). TBLSTX results of uORF sequence show highly conserved sequence of this region existing within different genes, and that the alfalfa uORF sequence shares great similarity to many gene sequences in other species, like *Arabidopsis* and rice (see section **4.3.1**). Based on the comparison of the similarity between the similar homeobox gene sequences in other species against the alfalfa HD-Zip gene (*Mfhb-1*), Arabidopsis *thaliana* HD-Zip transcription factor gene Athb-1 was found to be one of the most closely matched sequences. Analysis of TBLASTP results of the 29

bp alfalfa uORF shows greatest similarity to *A. thaliana* homeobox gene Athb-1 mRNA with 86% identities (25/29) and 96% positives (28/29) (Expect = 2.8e-07, P = 2.8e-07) in reading frame 1. The results of TBLASTP of full length HD-Zip gene revealed that the query sequence covering the region of 410-1255 bp, which almost includes the whole region of HD-Zip coding sequence, exhibits high similarity to *Arabidopsis thaliana* HD-Zip transcription factor gene Athb-1 (1302 bp in length) with 56% Identities (164/288), 72% Positives (210/288) (Expect =6.3e-88, Sum P (2) + 6.3e-88). All these results indicate that alfalfa HD-Zip gene (Mfhb-1) in this study is a close homologue of the *Arabidopsis thaliana Athb-1* homeobox gene.

Structural analysis of the alfalfa HD-Zip protein sequence revealed that, like other HD-Zip genes such as *Athb-1* (*Arabidopsis*) (Ruberti *et al.*, 1991) and *CHD1* (carrot) (Kawahara *et al.*, 1995), the homeodomain of this alfalfa HD-Zip gene also includes 3 helices (helix 1-helix 2-turn-helix 3) and is followed by a leucine zipper region (Figure 4.10). In the homeodomain region, Mfhb-1 shows considerable similarity to CHB1, particularly, both alfalfa HD-Zip gene (*Mfhb-1*) and *Arabidopsis* HD-Zip gene Athb-1 perfectly matches each other in helix 2-turn-helix 3 region. Various results from other studies accumulated over the last decade have shown that HD proteins function as transcription regulators and that the HD is responsible for sequence-specific binding to DNA via a helix-turn-helix motif (Gehring, 1992; Laughon, 1991; Scott *et al.*, 1989). The functions of Mfhb-1 and Athb-1 proteins most likely involve the same mechanisms in the regulation of transcription. Several studies of HD genes from plants have suggested that their products have DNA-binding activities and function in the regulation of plant growth (Schena *et al.*, 1993; Sessa *et al.*, 1993). CHB1 has been found to be involved in somatic embryogenesis in carrot. Ectopic expression of Athb-1 in tobacco suggested that Athb-1 is a transcription activator involved in leaf development.

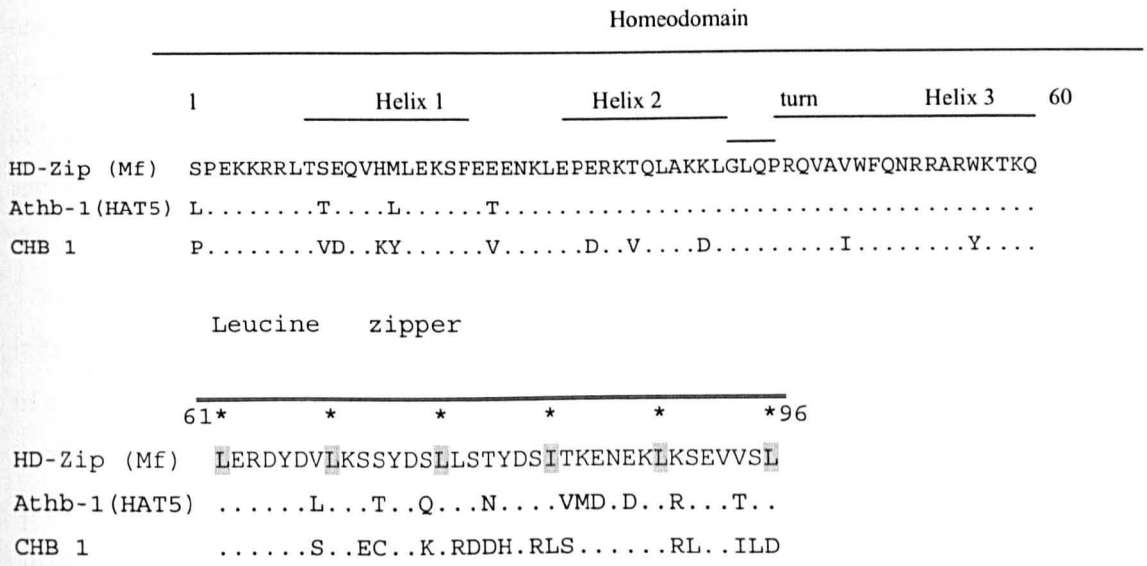

Figure 4.10 Comparison of the amino acid sequences of HD-Zip regions encoded by alfalfa HD-Zip gene (Mf) with HD proteins from *Arabidopsis* (Athb-1) and carrot (CHB1). Dots indicated identical amino acids in alfalfa HD-Zip gene (*Mfhb-1*) and other sequences. The positions of leucine (or isoleucine) residues in the leucine zipper motif are indicated by asterisks.

4.4.4 Fused cotyledon formation linked to the level of 2,4-D induction

During the regeneration of transgenic plants carrying Construct 4 (sense HD-Zip coding sequence) or 5 (sense full length HD-Zip) via the MSH system, a cup-shaped fused single cotyledon was formed (Figure 4.5) instead of the bilateral or multilateral cotyledon which typically developed during the MSH procedure. Unlike the normal bilateral or multilateral cotyledons, those cup-shaped fused cotyledon could not separate and develop shoots in later stages. The application of an elevated level of 2,4-D during the induction period in the MSH procedure, or to callus, permitted the bottle-shaped embryos to separate on the top to form bilateral or multicotyledons, and to develop shoots, with no cup-shaped fused cotyledons being produced. These results suggest that the formation of cup-shaped fused cotyledons is related to the level of 2,4-D during the early stages induction. Levels of 2,4-D below the minimum required for normal bilateral or multilateral cotyledon formation may not be sufficient for the separation of the apical pole of globular stage embryos,

resulting in the formation of cup-shaped fused cotyledons. Therefore, the level of 2,4-D applied in the induction stage of the MSH process is important for the formation of normal cotyledons during the early stages of somatic embryo development. Several studies have revealed that polar transport of auxin is a prerequisite for normal morphogenesis beyond the globular stage (Schiavone and Cooke, 1987; Liu *et al.*, 1993; Hadfi *et al.*, 1998). Treatment of early globular stage embryos of Indian mustard with auxin transport inhibitors induced the formation of fused cotyledons (Liu *et al.*, 1993). Zygotic embryos of *pin 1-1* mutants of *Arabidopsis*, which have reduced auxin transport, tend to develop a continuous cone of cotyledon tissue instead of two distinct cotyledons (Okada *et al.*, 1991). All of this evidence indicates that the formation of a fused cotyledon is linked to auxin transport and suggests a hypothesis that the ectopic or over-expression of the alfalfa HD-Zip transcription factor gene in this study may reduce auxin transport during the early stage of somatic embryogenesis.

Cup-shaped fused cotyledons have also been observed in the mutants of certain genes involved in establishing the shoot apical meristem (SAM) and separating cotyledons. e.g., in the *Arabidopsis* double mutant of *CUC1* and *CUC2*. *CUC1* and *CUC2* are thought to act upstream of *STM* (Takada *et al.*, 2001), a gene of the *knotted* class of homeodomain transcription factor (Long *et al.*, 1996). *STM* and *CUC2* are expressed in a stripe of cells across the top of the late globular embryo, forming the presumptive SAM (Aida *et al.*, 1999; Long *et al.*, 1996). *CUC1* and *CUC2* each encode NAC domain-containing proteins based on sequence similarity to the *NO APICAL MERISTEM* (*NAM*) gene in *Petunia* (Aida *et al.*, 1997; Takada *et al.*, 2001). *CUC1/ CUC2* have a role in establishing the SAM and separating cotyledons (Souer *et al.*, 1996). *CUC1* and *CUC2* double mutant seedlings completely lack an embryogenic SAM, and the two cotyledons are fused along both edges, resulting in a cup-shaped cotyledon. In my study, formation of cup-shaped fused cotyledons from transformed plant materials with Construct 4 and 5 produced by the normal MSH procedure mimics the phenotype of *CUC1* and *CUC2* double mutation. The lack of development of shoots from the cup-shaped cotyledons also suggests that SAM was not formed in these plants. However, because successful regeneration of alfalfa HD-Zip gene transgenic plants occurred by extension of the 2,4-D induction period, over-expression of this HD-Zip gene neither resulted in a genotyped lack of an embryogenic

SAM nor cup-shaped fused cotyledons. These observations suggest that the failure to develop a SAM during formation of the cup-shaped fused cotyledons from plant materials transformed with Construct 4 or 5 can be manipulated by the level of 2,4-D.

4.4.5 Factors that contributed to the success of the modified MSH system

The regeneration of alfalfa leaf explants *via* direct somatic embryogenesis in the MSH procedure could be altered by manipulation of the plant growth regulator levels in the medium. Using this regeneration system, many alfalfa transgenic plants transformed with several different constructs have been obtained efficiently, including *Arabidopsis cdc2a, CycB1;*1, *cyc3a* promoter::*gusA* fusions, CaMV35S promoter::*gusA* fusion (Shao *et al.*, 2000), and various *Bvcrk1* promoter fragment:: *gusA* fusions (see Chapter 2). The MSH system has proved to be a suitable regeneration system for alfalfa plant transformation with several different constructs. Many factors contribute to the success of the MSH procedure. The plant growth regulator (2,4-D) is an essential component of the system and appears to play an important role in the activation of cell division and subsequent reprogramming of direct somatic embryogenesis. In this study, the alfalfa explants transformed with different HD-Zip gene constructs required different levels of 2,4-D induction. For example, regeneration of transgenic plants transformed with constructs 4 and 5 required an extended 2,4-D treatment. In addition, the integration of half strength MS medium period into the development stages (maintaining the subculture on MS medium) has been proved to significantly improve the regeneration of whole plants *via* MSH regeneration system for alfalfa transformation.

4.4.6 The importance of optimal 2,4-D induction during somatic embryogenesis

2,4-D induction was shown in Chapter 3 to be an important component of the procedure for direct somatic embryogenesis in liquid medium. Optimal levels of 2,4-D induction are essential to achieve successful somatic embryogenesis. Analysis of the results of the somatic embryogenesis experiments in this study indicated that the different transformants had different requirements regarding the duration of the 2,4-D treatment for high yield

embryo production. A 26-day 2,4-D induction period appeared to be optimal for plants containing Constructs 2 and 3 and the control *Bvcrk1*, whereas transformants carrying Constructs 4 and 5 required an 18-day 2,4-D induction period for optimal somatic embryo formation (Figure 4.9).

Observations during the regeneration of these transgenic plants via MSH system suggested that a period of at least 12 days 2,4-D induction was required for regeneration of Construct 4 and 5 transgenic plants, whereas 10 days 2,4-D treatment in the standard MSH system was found to be sufficient to regenerate Construct 2 and 3 transformants. Thus, the transgenic plants showed different responses to 2,4-D both during their original regeneration on solid medium, and in subsequent experiments in liquid medium.

4.4.7 The role of the HD-Zip gene during direct somatic embryogenesis

The results from suspension culture experiments of direct somatic embryogenesis (Fig 4.9) revealed different patterns of direct somatic embryogenesis among the four lines of transformants. In general, those transformed with Constructs 4 and 5 presented a similar pattern, with an optimal 2,4-D induction period of 18 days for achieving the highest numbers of embryos. Shorter or longer induction periods resulted in a reduced yield of embryos. A similar pattern between Construct 4 and 5 was also observed during regeneration of the transgenic plants. Interestingly, transformants of Construct 3 showed a pattern similar to the control *Bvcrk1* transformants, with an optimal induction period of 26 days. Shorter induction periods (10 or 18 days) led to a reduction in the number of embryos produced. Plants transformed with construct 2 exhibited a unique pattern, with lower yields of embryos overall; 26 days induction only produced a slightly higher number of embryos than with 10 or 18 days induction. The highest yield of embryos production was observed with Construct 5 transformants among all the tested plants. These observations indicate that over-expression of HD-Zip gene fragment sequences (uORF, CDS, and uORF + CDS) resulted in an overall changed patterns of somatic embryogenesis. The analysis of the patterns of somatic embryogenesis suggest that the full length HD-Zip gene plays a positive role during somatic embryogenesis, and that the uORF appeared to functions as an enhancer of the expression of HD-Zip CDS.

However, the observation that a shorter 2,4-D induction period is required for optimal somatic embryogenesis in suspension cultures appears to be at odds with the requirement for a prolonged 2,4-D treatment during the regeneration of plants transformed with Constructs 4 and 5. The exact reasons are unclear. One possible reason could be due to the response to solid (during regeneration of transformants) as opposed to liquid medium (during direct somatic embryogenesis in suspension culture). Under the conditions with those two different systems, the regulation of over-expression of Construct 4 or 5 may be different. If so, the impact of the over-expression produced from those two different systems possibly results in conflicting observations. Information about over-expression of Construct 4 and 5 in those two different systems is required for further analysis.

In the case of construct 3, it was expected that there might be a failure in embryo formation, or lower production of embryos or regeneration of abnormal phenotypes. However, a similar pattern of somatic embryogenesis was found between Construct 3 and the control, suggesting that reduced expression of the HD-Zip gene (*Mfhb-1*) as a consequence of antisense expression has no significant effect on somatic embryogenesis. Furthermore, this observation indicates that the *Mfhb-1* is not an essential gene in the control of somatic embryogenesis. This point is supported by evidence from this study, in that somatic embryos could be formed and developed with a normal phenotype during the regeneration of transgenic plants with Construct 3. Alternatively, the antisense expression of HD-Zip gene in this study was not sufficient to knock out the target gene, i.e. the CaMV35S promoter may not induce sufficient expression of the anti-sense HD-Zip to suppress the endogenous HD-Zip gene transcript in these cells. Further work is required before the conclusion can be reached.

CHAPTER 5 GENERAL DISCUSSION

5.1 Optimisation of *Agrobacterium*-mediated transformation of alfalfa

Alfalfa is one of the most important crops cultivated worldwide. It has long been the focus of plant breeding efforts because of its importance as a high quality forage crop (Hill *et al.*, 1991). It has served as a model species for the genetic manipulation of other members of the family *Leguminosae* due to its ability to form nitrogen-fixing root nodules in symbiosis with *Rhizobium* species. A better understanding of the molecular genetics of *Rhizobium*-legume symbiosis will make a significant contribution to improvements in agriculture and the environment (Schultze *et al.*, 1994; Long, 1996; Trinh *et al.*, 1998). Alfalfa genetic engineering has proved to be a useful research tool and offers the potential to be a powerful strategy for alfalfa improvement. Alfalfa has been successfully transformed with both *Agrobacterium* vectors and by particle bombardment. In most cases *Agrobacterium*-mediated transformation yields a higher frequency of the desired simple, single copy insertions (McCaslin, 2002).

Alfalfa has also been developed as an experimental model for the molecular characterisation of somatic embryogenesis (Dudits *et al.*, 1991, 1995) which offers opportunities for the regeneration of genetically modified plants by genetic transformation. Direct somatic embryogenesis proceeds from cells within organized tissue which already have embryogenic potential. The cells in differentiated tissue which have the potential to become embryogenic directly have been described by some authors as pre-embryogenic determined cells (PEDCs) (Williams and Maheswaran, 1986). PEDCs require external stimuli only to induce the onset of cell division and the expression of embryogenesis. The capacity of cells to respond in this way to induction signals (plant growth regulators and wounding) has been termed embryogenic competence.

In this study, a direct somatic embryogenesis system based on the use of solid MSH medium was used to regenerate transgenic alfalfa plants following *Agrobacterium*-mediated transformation (Shao *et al.*, 2000). In the modified MSH system used in this study, inoculated leaf and petiole explants were placed on MS medium supplemented with 2,4-D and kinetin for 10 days for the induction of somatic embryos, then on MS medium

for further development. The successful regeneration of transgenic alfalfa plants with all of the different constructs used in this study indicated that the MSH procedure is a suitable regeneration system for alfalfa transformation. It also suggested that several key elements contributed to the optimisation of alfalfa transformation and subsequent regeneration of the transgenic plants:

1. Choosing young alfalfa tissues appeared to be necessary to ensure an efficient transformation and plant regeneration. This is because young tissue is normally easier to be infected by *Agrobacterium* and the population of PEDCs is generally higher in young tissues than that in old ones.
2. The efficient control of bacterial overgrowth after co-cultivation period appeared to be an essential step to a successful transformation. The transformation of alfalfa plants with different constructs has shown that both cefotaxime and Timentin are suitable antibiotics to control overgrowth of both EHA101 and LBA4404 in the MSH transformation system. For cefotaxime, suitable concentrations ranged from 150 to 350 mg L^{-1}, while Timentin for 150 to 250 mg L^{-1}, was sufficient to control bacterial overgrowth. Higher concentrations of these antibiotics were harmful to the plant cells and resulted in poor regeneration, or even to kill the plant cells. Data from the MSH system in this study indicated that the optimal concentrations for alfalfa transformation are 300 mg L^{-1} cefotaxime and 200 mg L^{-1} Timentin. Carbenicillin is not an efficient antibiotic for the control of the EHA 101 strain.
3. The regeneration of transgenic plants with Constructs 2, 3, 4 and 5 showed that the integration of a half strength MS medium period into the development stages (maintaining the subculture on MS medium) significantly improved the regeneration of alfalfa plants.
4. Adding kanamycin into the medium after the induction period rather than after co-cultivation with *Agrobacterium* (as is normally done) appeared to improve somatic embryo development and whole plant regeneration. A period allowing the kanamycin resistant gene to express may be important to ensure that the transformed cells survive the kanamycin selection.
5. Comparison of transformations carried out with the several different constructs used in this study showed that the period of 2,4-D induction required for each individual

construct may be different. These results indicated that it is necessary to consider the levels of 2,4-D applied in MSH system to meet the individual requirements for the transformation with each construct and to ensure successful somatic embryogenesis leading to the efficient production of the normal plants.

5.2 The regulation of cell division during direct somatic embryogenesis

The reactivation of the cell cycle in differentiated plant cells under the influence of external stimuli is one of the basic features of the initiation of somatic embryogenesis. The artificially induced series of cell divisions precedes a switch from the somatic to the embryogenic cell type (Dudits *et al.*, 1995). Acquisition of embryogenic competence closely relies on dedifferentiation, a process whereby existing transcriptional and translational profiles are erased or altered in order to allow cells to set new developmental programmes. The activation of cell division is required to maintain the dedifferentiated cell fate, as well as for somatic embryo differentiation (for review, Fehér *et al.*, 2003). On the basis of these considerations, molecular studies on somatic embryogenesis have been focused on the regulatory mechanisms of the cell cycle (Dudits *et al.*, 1991; 1995). In the light of a number of available experimental results, a link may be proposed between accelerated activation of the cell cycle and the embryogenic process in somatic cells. Thus embryogenic induction cannot be understood without information about the regulation of the cell cycle (Dudits *et al.*, 1991; 1995).

Cell cycle inhibitors are powerful and effective tools for studying the biochemical events of the cell cycle in plants. Oryzalin has strong binding affinity for plant tubulins and inhibits microtubule polymerization. It was shown to arrest cells at metaphase. Hydroxyurea (HU) is another frequently used cell cycle inhibitor. It inhibits the activity of ribonucleotide diphosphate reductase, thus depriving the cells of newly synthesized deoxyribonucleotide triphosphates, consequently preventing DNA replication (Young and Hodas, 1964). HU therefore blocks G1/S progression. In this study, both oryzalin and HU were used to investigate the relationship between cell division and embryo formation during the early induction.

Previous studies have revealed that *Arabidopsis cdc2* could be a good marker for detection of cells that are "competent for division", while *CycB1;*1 could be used as a marker for actively dividing cells in alfalfa (Shao's PhD thesis, 2000). In our experiments, the activity of cell division cycle genes during direct somatic embryogenesis was investigated using transgenic alfalfa plants containing the *gusA* gene under the control of the *Arabidopsis cdc2a* and *CycB1;*1 promoters. The results showed that after induction, the expression pattern of both genes was similar, in that GUS activity was first observed after 2-3 days (*cdc2* was expressed slightly earlier than *CycB1;*1) tracing the lines of the leaf vascular elements and subsequently spreading throughout the tissue by 5 days. The similarity continued until 15 days. By 15 days, in the case of *cdc2*, the GUS staining was more intense in the globular pro-embryos, but activity could still be observed in the surrounding cells, while *CycB1;*1 activity remained high in the pro-embryo cells, but had disappeared from the surrounding cells by this stage. These results indicated that the reactivation of cell division is an early event in the induction process, and appears to occur in all of the leaf cells. Continued cell division in specific clusters of small cells is required for embryo development.

In another approach, cell cycle inhibitors were used to examine the role of cell division during somatic embryogenesis in transgenic plants containing the *gusA* gene under the control of *Arabidopsis CycB1;*1 promoter. When leaf explants were cultured with B_5IV induction medium containing 10 mM HU, only a very low level of *gusA* expression could be detected after 14 days induction and no embryos were found after 59 days in developmental medium (Table 2.5). This result together with above investigation demonstrated that the activation of cell division and the maintenance of that division activity in certain cells is necessary for the induction and subsequent development of embryos. It is possible that the hydroxyurea block in G_1/S phase prevents the accumulation of particular products that might be essential for somatic embryogenesis.

Observations from the suspension cultures in B_5IV induction medium containing 15 μM oryzalin showed that a high level of *CycB1;*1 expression was found from 5 to 14 days induction period, which then declined. Interestingly, a greater number of embryos were observed at an earlier stage during this treatment than in the control. The results suggested that blocking cell division activity itself during the induction period does not prevent

somatic embryogenesis, indeed it appears to enhance it. One explanation is that the M phase block leads to an accumulation of products synthesised in G2 phase, which are involved in directing embryo formation. Alternatively, it may be that the combination of 2,4-D with oryzalin mimics the optimal balance of cell division activation/inhibition proposed by the model of Pasternak *et al.*, 2002 (Chapter 2) for the induction of somatic embryogenesis in alfalfa.

5.3 The role of 2,4-D in different systems

Among different auxins, 2,4-D is the most commonly applied for somatic embryogenesis. In our study, 2,4-D plays an important role in activating cell division and triggering somatic embryogenesis in alfalfa (see chapter 2 and 3). However, the effect of 2,4-D on cell division was different in different plant systems. Thus 2,4-D treatment resulted in different expression patterns of the cell cycle gene promoters in alfalfa and tobacco. The results indicated that 2,4-D may play a different role of in stimulating of cell division in these different systems. This may also reflect the different ability of different tissues to respond to 2,4-D induction in different species. In alfalfa, 2,4-D induction caused some cells to divide rapidly, forming small, round, cytoplasm-rich cells that subsequently develop into embryos. In tobacco, only elongated cells formed and no embryos were produced from these experiments. These observations indicated that the level of 2,4-D induction used here, which is normally suitable for induction of somatic embryogenesis in alfalfa, is not sufficient to trigger cell division in tobacco. High concentrations of 2,4-D have been reported to inhibit cell elongation and subsequently promote cell division in carrot (Lloyd *et al.*, 1980) and alfalfa leaf protoplast systems (Pasternak *et al.*, 2002). Taken together with the analysis of cell division patterns in both alfalfa and tobacco from this study (Chapter 3), it can be suggested that the failure of 2,4-D to induce somatic embryogenesis in tobacco is related to a more fundamental difference between alfalfa and tobacco in the response to 2,4-D in activation of cell division.

5.4 The gene expression pattern during the early induction of somatic embryogenesis

More than 100 different clones including identified and some non-identified clones were examined in this study. This collection of gene sequences isolated by subtractive cloning from alfalfa explants encodes a range of proteins including transcription factors (e.g., HD-Zip, bZIP), signal transduction components, cytoskeletal proteins, membrane transport proteins, wound and stress-related proteins, electron transport proteins, kinases, the phosphatase PP2C, and auxin-induced genes. The largest group of genes encodes ribosomal proteins and other proteins involved in ribosome biosynthesis, translation and post-translational modification. Similar genes have also been identified in other systems (e.g. in carrot, cell wall proteins, pathogenesis-related (PR) proteins, heat-shock proteins, ribosomal proteins, etc.) (Lin *et al.*, 1996). The different systems may share in common a similar group of genes which are involved in somatic embryogenesis. Therefore, data generated from this study may provide useful information for studies of other systems, and contribute to a comprehensive analysis to reveal the characteristic patterns of gene expression during the early events of somatic embryogenesis.

In order to determine the temporal expression of these clones during the early induction period, dot blot hybridization was carried out. Dot blot hybridization is a feasible, rapid method for checking the expression of a large number of genes during embryogenesis or plant development. Results from our studies showed that most of those clones were significantly expressed after 7 days induction with 2,4-D, but most of them were not detectable after 7 days if only a short (10 min) induction treatment with 2,4-D was given. These results indicated that a prolonged 2,4-D induction period was required for the expression of most of the clones which might possibly be involved in the initiation of somatic embryogenesis. Our observations that only a very short 2,4-D induction treatment (minutes) is required to activate cell division and callus formation but that at least 2-3 days induction is required for embryo formation are of relevance here. An intermediate period of 2,4-D treatment led to root formation in liquid culture (as in Figure 2.10, D). The implication of these results is that 2,4-D is the primary signal that triggers the expression of most of the genes in the subtractive libraries. However, the length of exposure is critical in determining which genes are expressed. The fact that different times of 2,4-D exposure also affects the developmental competence of cells in the leaf explants (callus, root or embryo) suggests a model in which different groups of genes are

expressed after different times of exposure, leading to different developmental pathways. This is indicative of the multiple roles of 2,4-D in the induction of somatic embryogenesis. In the alfalfa direct somatic embryogenesis system used in this study, somatic embryos were formed on leaf pieces as a result of the combined action of wounding and 2,4-D. Comparing with our previous studies, however, many of the identified genes could be induced by wounding or 2,4-D alone (Shao, 2000). Therefore, further analysis becomes necessary to elucidate the potential significance of the protein products of these genes during somatic embryogenesis.

5.5 The role of the alfalfa HD-Zip transcription factor Mfhb-1

5.5.1 The HD-Zip transcription factor may be linked to auxin transport

During the transformation of alfalfa with the HD-Zip transcription factor gene, regeneration of the transgenic plants appeared to be very difficult. Only cup-shaped fused cotyledons formed rather than the bilateral cotyledons normally produced from the MSH system. These cup-shaped fused single cotyledons could not develop into normal plantlets. When applying an increased 2,4-D induction treatment, bottle-shaped embryos could separate on the upper region of the explant to form bilateral or multicotyledons and develop shoots, with no cup-shaped fused cotyledon being produced. These results suggest that cup-shaped fused cotyledon formation was related to the level of 2,4-D induction in the early stages of the regeneration procedure. Lower levels of 2,4-D than the minimum required for induction of normal bilateral or multilateral cotyledon formation could block the polarisation of auxin distribution at the globular stage of embryo development and subsequently result in a failure to initiate a SAM. As a result, the cup-shaped fused cotyledon was formed. The requirement for increased 2,4-D induction after transformation with the sense HD-Zip constructs indicated that the HD-Zip gene might be involved in the auxin response. Several other studies have revealed that polar transport of auxin is a prerequisite for normal morphogenesis (Schiavone and Cooke, 1987; Liu *et al.*, 1993; Hadfi *et al.*, 1998) beyond the globular stage. Treatment of early globular stage embryos of Indian mustard with auxin transport inhibitors induced the formation of fused

cotyledons (Liu *et al.*, 1993). Zygotic embryos of *pin 1-1* mutants of *Arabidopsis*, which have reduced auxin transport, tend to develop a continuous cone of cotyledon tissue instead of two distinct cotyledons (Okada *et al.*, 1991). The *gnom* mutant phenotype, which forms partially or completely fused cotyledons (Meinke, 1985; Mayer *et al.*, 1993), can be mimicked by altering auxin transport or response in the experimentally accessible early embryo of the closely related species *Brassica juncea* (Hadfi *et al.*, 1998). Thus, the formation of fused cotyledons is linked to auxin transport and furthermore suggests a hypothesis that the alfalfa HD-Zip transcription factor gene in this study may be involved in reducing auxin polar transport during the early stages of somatic embryogenesis.

5.5.2 The HD-Zip transcription factor involved the control of plant patterning

Well-documented evidence implicates auxin as playing a major role in embryogenesis, providing positional information for the co-ordination of correct cellular patterning from the globular stage onwards (Normanly and Bartel, 1999). Vascular tissue formation follows the flow of auxin (Aloni, 1987; Mattsson *et al.*, 1999). Auxin controls much more of post-embryonic development, especially plant architecture, through the modulation of meristem activity and cell expansion in response to environmental factors (Hobbie, 1998). The phenotype of the cup-shaped fused cotyledon observed in this study appeared to be due to a failure of shoot apical meristem (SAM) development. The reason for failed development of the SAM is possibly because overexpression of HD-Zip gene leads to an aberrant polar auxin distribution so that the SAM could not develop. Taken together with the discussion from 5.5.1, it can be hypothesized that this alfalfa HD-Zip transcription factor is involved in the control of plant patterning via the auxin transport pathway during the early stages of somatic embryogenesis.

5.6 Future work

5.6.1 Using RT-PCR technique to determine the efficiency of anti-sense transformation

Antisense technology is an important method to study transcription factors in plants. This method used RNA interference (RNAi) interactions to effectively knockout the host analogue of the transcription factor, therefore the resultant plant will grow with a reduced level or complete absence of the transcription factor in question. The use of anti-sense technologies are therefore powerful tools in determining transcription factor function. However, there are limitations to this method in that it is difficult to assess the extent of suppression within the plant and sometime it may not efficiently knockout the target gene. In this project, sense and antisense techniques were used to investigate the biological function of the alfalfa HD-Zip transcription factor gene *Mfhb-1*. Surprisingly, observations from the regeneration of the HD-Zip-antisense transgenic plants and subsequent assay of direct somatic embryogenesis showed that there is not much difference found between antisense transgenic plants and the non-transformed plants, while the sense transgenic plants exhibited significant differences. Several questions can be asked:

- Why was no difference found between antisense transgenic plants and non-transformed plants?
- Does the lower expression of the HD-Zip transcription factor gene Mfhb-1 really not affect the function of the plant?
- Does the antisense method work effectively in this case?

To answer these questions, assays to determine the efficiency of antisense transformation become necessary to be carried out. RT-PCR is a sensitive and highly accurate technique to detect the expression of the gene of interest in plant and therefore qualifies to meet this purpose.

5.6.2 Further determination of the role of alfalfa HD-Zip transcription factor

5.6.2.1 Scale-up of somatic embryogenesis of HD-Zip transgenic alfalfa plants

Due to the limitation of facilities and plant materials caused by moving laboratories, the experiments on direct somatic embryogenesis in HD-Zip transformed plants had to be reduced in scale for this study. Data obtained from small scale experiments may not accurately reflect the true characteristic of the plants. Re-scaled experiments in future may

provide more accurate information which may significantly contribute to reveal the biological function of the HD-Zip transcription factor gene in alfalfa.

5.6.2.2 Determination of HD-Zip gene expression patterns by Northern hybridisation

To investigate the role of the alfalfa HD-Zip gene and subsequently characterise its function, information from molecular analysis seems to be necessary. Northern blot hybridisation assays will provide detailed gene expression profiles. Integrated with the data from re-scaled somatic embryogenesis experiments, the relationship between the HD-Zip gene expression and somatic embryogenesis may be determined.

5.6.2.3 Application of auxin transport inhibitor

In order to verify the hypothesis that the HD-Zip transcription factor is possibly involved in auxin transport during the early induction of somatic embryogenesis, application of other auxins and auxin transport inhibitors to HD-Zip transgenic explants could be investigated.

5.6.2.4 Determination of the level of endogenous hormones in HD-Zip gene transgenic plants

During the last few years, a large body of experimental observations has accumulated on the central roles of endogenous indoleacetic acid (IAA) and abscisic acid (ABA) levels during the early phases of embryogenesis (for review, Fehér *et al.*, 2003). Higher endogenous IAA concentration has been shown to be associated with increased embryogenic response in various species/explants (Rajasekaran *et al.*, 1987; Ivanova *et al.*, 1994; Michalczuk and Druart, 1999; Jimenez and Bangerth, 2001a, b, c). High IAA and low ABA levels were observed in fast embryogenic lines compared with low embryogenic and non-embryogenic lines in alfalfa (*Medicago falcata* L.) (Ivanova *et al.*, 1994). In carrot cells, exogenous 2,4-D stimulated the accumulation of large amounts of endogenous IAA (Michalczuk *et al.*, 1992a, b). These authors hypothesised that

embryogenic competence of carrot cells is tightly associated with the several-fold increase in endogenous IAA levels due to the presence of 2,4-D. It was suggested that 2,4-D acts indirectly by disturbing endogenous auxin metabolism, and that the direct auxin effect of 2,4-D is less significant (for review, Fehér *et al.*, 2003).

During the regeneration of the transgenic plants with sense HD-Zip or full length HD-Zip gene constructs, the presence of the transgene appeared to affect auxin responses. Longer 2,4-D induction was required for regeneration of HD-Zip transgenic plants compared to those with other constructs (e.g. *Bvcrk1* construct). This gene may physiologically affect the levels of endogenous hormones in transgenic plants. It was hypothesized that over expression of the HD-Zip gene in alfalfa alters the endogenous hormone balances which effects the ability to respond to a 2,4-D induction treatment and subsequent embryogenic expression. In order to reveal the relationship between the HD-Zip gene expression and the levels of endogenous hormones (e.g., IAA and ABA) during the induction of somatic embryogenesis, it would be interesting to determine the levels of endogenous hormones.

In particular, it will be of great interest to investigate whether the involvement of the HD-Zip gene in modulating auxin responses ultimately plays a role in the control of embryogenic competence of alfalfa cells.

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Appendix

The overall information from BLASTP searching results of selected clones

a) bZip

The translated protein sequence submitted to BLAST searching:
GGRPGRYKLRLQSLEQQSQLKDALNETLNGEVRRLRHTVAELGGESALS GLMARQLAINQQMFQAQHQQPNQLRNFQPQNSVSQEETQTQSQQHIQR NHEFQSKHQNGKTTA

BLASTP Results:

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BLASTP 2.0MP-WashU [23-May-2003] [decunix5.0a-ev56-IP32LF64 2003-05-
23T14:40:20]

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USA.
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Reference:  Gish, W. (1996-2003) http://blast.wustl.edu

Query=  Sequence
        (112 letters)

Database:  uniprot
           1,403,392 sequences; 447,949,266 total letters.
Searching....10....20....30....40....50....60....70....80....90....10
0% done


Smallest

Sum
                                                                  High
Probability
Sequences producing High-scoring Segment Pairs:                    Score
P(N)       N

UNIPROT:Q8S5V5 Q8S5V5 Putative bZIP transcription factor.          165
5.6e-11    1
UNIPROT:O24181 O24181 RF2a.                                        148
2.9e-09    1
UNIPROT:O81998 O81998 Transcription factor VSF-1.                  145
9.0e-09    1
UNIPROT:Q43530 Q43530 Vsf-1 protein.                               145
9.0e-09    1
UNIPROT:Q8H1F0 Q8H1F0 Hypothetical protein.                        141
3.4e-08    1
UNIPROT:Q8RXK4 Q8RXK4 Hypothetical protein.                        141
3.4e-08    1
UNIPROT:Q9T0J7 Q9T0J7 Hypothetical protein (Transcription...       141
3.5e-08    1
UNIPROT:Q8LMJ4 Q8LMJ4 Putative transcription factor.               136
6.7e-08    1
```

UNIPROT:Q8LN44 Q8LN44 Putative transcription factor. 136
7.0e-08 1
UNIPROT:PF21_ARATH Q04088 Possible transcription factor P... 135
9.0e-08 1
UNIPROT:O22873 O22873 Putative bZIP transcription factor. 131
2.1e-07 1
UNIPROT:O23726 O23726 B-Zip DNA binding protein. 131
2.1e-07 1
UNIPROT:Q9SIG8 Q9SIG8 Putative bZIP transcription factor ... 129
6.2e-07 1
UNIPROT:Q8VWG0 Q8VWG0 AtbZIP transcription factor (Simila... 124
1.0e-06 1
UNIPROT:Q8LDZ9 Q8LDZ9 Transcriptional activator RF2a, put... 125
1.2e-06 1
UNIPROT:Q9LNE0 Q9LNE0 T21E18.12 protein (AtbZIP transcrip... 125
1.2e-06 1
UNIPROT:Q9M9Z1 Q9M9Z1 F4H5.7 protein. 124
1.5e-06 1
UNIPROT:Q84LF3 Q84LF3 bZIP-like protein. 116
1.8e-06 1
UNIPROT:AAR28765 AAR28765 BZIP transcription factor RF2b. 114
1.2e-05 1
UNIPROT:BAC84100 BAC84100 Putative transcription activato... 108
4.3e-05 1
UNIPROT:Q7XUJ7 Q7XUJ7 OSJNBa0067K08.2 protein. 111
4.6e-05 1
UNIPROT:O49220 O49220 Shoot-forming PKSF1. 107
7.5e-05 1
UNIPROT:Q9M5N9 Q9M5N9 VirE2-interacting protein VIP1. 103
0.00012 1
UNIPROT:Q8LDQ9 Q8LDQ9 VirE2-interacting protein VIP1. 104
0.00016 1
UNIPROT:Q9LRC7 Q9LRC7 bZIP transcriptional activator RSG. 104
0.00017 1
UNIPROT:Q9MA75 Q9MA75 Putative transcription factor (Puta... 103
0.00021 1
UNIPROT:Q8VWR2 Q8VWR2 AtbZIP transcription factor. 97
0.00052 1
UNIPROT:Q86GH1 Q86GH1 Pol protein. 105
0.00087 1
UNIPROT:Q7XXC1 Q7XXC1 OSJNBa0027001.8 protein. 97
0.0013 1
UNIPROT:O76940 O76940 GAGA factor class A-isoform. 98
0.0015 1
UNIPROT:Q86FU9 Q86FU9 Mastermind (Fragment). 94
0.0015 1
UNIPROT:Q86AF2 Q86AF2 Similar to Dictyostelium discoideum... 88
0.0017 2
UNIPROT:Q9BLX2 Q9BLX2 STATc protein. 100
0.0017 1
UNIPROT:BAC85523 BAC85523 CDNA FLJ41049 fis, clone PROST2... 97
0.0018 1
UNIPROT:Q869L1 Q869L1 Similar to Dictyostelium discoideum... 103
0.0020 1
UNIPROT:Q86CU6 Q86CU6 Mastermind (Fragment). 93
0.0020 1
UNIPROT:Q7SH18 Q7SH18 Predicted protein. 93
0.0022 1

UNIPROT:CAE76500 CAE76500 Hypothetical protein B12J7.050. 93
0.0022 1
UNIPROT:Q7Z315 Q7Z315 Hypothetical protein DKFZp686O11215... 97
0.0024 1
UNIPROT:Q8N4P9 Q8N4P9 PAX transcription activation domain... 97
0.0028 1
UNIPROT:CAE45762 CAE45762 Hypothetical protein DKFZp686E0... 97
0.0031 1
UNIPROT:Q946W1 Q946W1 50kD gamma zein. 91
0.0033 1
UNIPROT:BAC85657 BAC85657 CDNA FLJ41606 fis, clone CTONG3... 97
0.0040 1
UNIPROT:IFA2_CAEEL O02365 Intermediate filament protein i... 94
0.0042 1
UNIPROT:O35858 O35858 Sex determining protein. 88
0.0045 1
UNIPROT:GBF_DICDI P36417 G-box binding factor (GBF). 94
0.0053 1
UNIPROT:Q9W4H2 Q9W4H2 CG12683 protein. 82
0.0057 1
UNIPROT:O35860 O35860 Sex determining protein. 87
0.0058 1
UNIPROT:Q86JP2 Q86JP2 Similar to Mus musculus (Mouse). si... 95
0.0064 1
UNIPROT:Q7S1P2 Q7S1P2 Predicted protein. 87
0.0077 1
UNIPROT:Q7N0H5 Q7N0H5 Nuclease sbcCD subunit C. 95
0.0079 1
UNIPROT:Q7Q109 Q7Q109 AgCP8788 (Fragment). 91
0.0082 1
UNIPROT:Q84SZ4 Q84SZ4 Unknow protein. 92
0.0083 1
UNIPROT:RAS1_CANAL Q9UQX7 Ras-like protein 1 (Ras homolog... 87
0.0087 1
UNIPROT:Q8NJR4 Q8NJR4 Ure2p. 89
0.0088 1
UNIPROT:Q960S0 Q960S0 LD38452p (CG11494-PA) (CG11494-PB). 92
0.0092 1
UNIPROT:Q86K33 Q86K33 Hypothetical protein. 92
0.0094 1
UNIPROT:Q9SKG1 Q9SKG1 Putative VSF-1-like b-ZIP transcrip... 86
0.0095 1
UNIPROT:Q7S071 Q7S071 Predicted protein. 87
0.011 1
UNIPROT:Q86GH2 Q86GH2 Pol protein (Fragment). 94
0.011 1
UNIPROT:Q23847 Q23847 Glutamine-asparagine rich protein (... 91
0.011 1
UNIPROT:Q86GH6 Q86GH6 Pol protein (Fragment). 90
0.012 1
UNIPROT:Q7PKF6 Q7PKF6 ENSANGP00000022683 (Fragment). 79
0.012 1
UNIPROT:O76853 O76853 SRF related protein. 88
0.012 1
UNIPROT:INVO_HYLLA P17941 Involucrin. 89
0.012 1
UNIPROT:NCO2_XENLA Q9W705 Nuclear receptor coactivator 2 ... 94
0.013 1

UNIPROT:Q86IS0 Q86IS0 Similar to Dictyostelium discoideum... 96
0.013 1
UNIPROT:Q9VSK3 Q9VSK3 CG7015 protein. 92
0.014 1
UNIPROT:Q9W6J4 Q9W6J4 Transcription factor clock. 80
0.014 2
UNIPROT:Q9V585 Q9V585 CG12933 protein. 85
0.014 1
UNIPROT:Q867Z5 Q867Z5 Gag protein. 83
0.015 2
UNIPROT:SP97_DICDI Q95ZG3 Spindle pole body component 97 ... 83
0.016 2
UNIPROT:Q9VE07 Q9VE07 CG6026 protein. 93
0.016 1
UNIPROT:YHY6_YEAST P38873 Hypothetical 175.8 kDa Trp-Asp ... 85
0.017 2
UNIPROT:SEU_ARATH Q8W234 Transcriptional co-repressor SEUSS. 90
0.018 1
UNIPROT:O77283 O77283 EG:EG0002.3 protein. 93
0.019 1
UNIPROT:Q8I3R4 Q8I3R4 Hypothetical protein. 89
0.020 1
UNIPROT:Q87G62 Q87G62 Hypothetical protein. 88
0.020 1
UNIPROT:Q8IRT3 Q8IRT3 CG2904-PB. 93
0.020 1
UNIPROT:Q95T56 Q95T56 GH20830p. 84
0.020 1
UNIPROT:P58462-2 P58462 Splice isoform B of P58462 88
0.021 1
UNIPROT:Q86L10 Q86L10 Similar to Vibrio vulnificus CMCP6.... 80
0.021 1
UNIPROT:FXP1_MOUSE P58462 Forkhead box protein P1 (Forkhe... 88
0.023 1
UNIPROT:Q9UVU4 Q9UVU4 Ras homolog type A. 83
0.023 1
UNIPROT:Q86AU2 Q86AU2 Similar to Dictyostelium discoideum... 87
0.024 1
UNIPROT:Q8IRM5 Q8IRM5 CG1343-PB. 86
0.026 1
UNIPROT:Q9VAP9 Q9VAP9 CG11873 protein. 86
0.026 2
UNIPROT:SLYX_HAEIN P44759 Protein slyX homolog. 53
0.026 2
UNIPROT:P73340 P73340 Chromosome segregation protein SMC1. 90
0.026 1
UNIPROT:Q8MYI6 Q8MYI6 Similar to Dictyostelium discoideum... 90
0.026 1
UNIPROT:Q7RWZ1 Q7RWZ1 Hypothetical protein. 88
0.026 1
UNIPROT:AAQ94949 AAQ94949 SMA-9 class B. 87
0.029 1
UNIPROT:AAS18267 AAS18267 TATA box binding protein (Fragm... 75
0.031 1
UNIPROT:AAQ75701 AAQ75701 TATA box binding protein (Fragm... 75
0.031 1
UNIPROT:Q9HG15 Q9HG15 Putative GAL4-like transcriptional ... 87
0.031 1

```
UNIPROT:Q94486 Q94486 ORF DG1040 (Fragment).                          86
0.032     1
UNIPROT:Q86A44 Q86A44 Similar to Dictyostelium discoideum...          85
0.033     1
UNIPROT:AAR97288 AAR97288 DIF insensitive mutant A.                   89
0.034     1
UNIPROT:Q86GH5 Q86GH5 Pol protein.                                    90
0.034     1
UNIPROT:Q8T2S4 Q8T2S4 Similar to Dictyostelium discoideum...          86
0.034     1
```

b) PP2C

The translated protein sequence submitted to BLAST searching:

SQVHAADDTPVSGGGLSQNGKFSYGYASSPGKRSSMEDFYETRIDGVDGE VVGLFGVFDGHGGARAAEYVKQNLFSNLISHPKFISDTKSAIADAYTHTD SEFLKSENNQNRDAGSTASTAILVGDRLLVANVGDSRAVICRGGNAIAVSR DHKPDQTDERQRIEDAGGFVMWAGTWRVGGVLAVSRAFGDRLLKQYV VADPEIQEEKVDSSLEFLILASDGLWDVVSNEEAVAMIKPIEDAEEAAKRL MKEAYQRGSSDNITCVVIRFLMNNQGSSSRNSSG

BLASTP results:

```
BLASTP 2.0MP-WashU [23-May-2003] [decunix5.0a-ev56-IP32LF64 2003-05-
23T14:40:20]

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USA.
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Reference:  Gish, W. (1996-2003) http://blast.wustl.edu

Query=  Sequence
        (283 letters)

Database:  uniprot
           1,403,392 sequences; 447,949,266 total letters.
Searching....10....20....30....40....50....60....70....80....90....10
0% done


Smallest

Sum

                                                                    High
Probability
Sequences producing High-scoring Segment Pairs:                      Score
P(N)       N

UNIPROT:Q8S8Z0 Q8S8Z0 Protein phosphatase 2C.                        1296
1.3e-131   1
UNIPROT:Q8RXV3 Q8RXV3 Hypothetical protein.                           1289
7.2e-131   1
UNIPROT:O81773 O81773 Hypothetical protein.                           935
2.2e-123   2
```

UNIPROT:Q8LAY8 Q8LAY8 Protein phosphatase 2C-like protein... 1189
2.8e-120 1
UNIPROT:Q7XR06 Q7XR06 OSJNBa0015K02.7 protein. 1163
1.6e-117 1
UNIPROT:CAE54579 CAE54579 OSJNBa0011F23.20 protein. 1163
1.6e-117 1
UNIPROT:Q9LEW5 Q9LEW5 Protein phosphatase 2C-like protein. 1133
2.4e-114 1
UNIPROT:Q8S3P1 Q8S3P1 Hypothetical protein. 1020
2.3e-102 1
UNIPROT:Q8VZN9 Q8VZN9 Hypothetical protein. 905
3.5e-90 1
UNIPROT:Q94AT1 Q94AT1 Putative phosphatase 2C. 839
3.5e-83 1
UNIPROT:Q8VZD9 Q8VZD9 AT5g53140/MFH8_8. 833
1.5e-82 1
UNIPROT:Q9LP12 Q9LP12 F9C16.6. 541
6.6e-80 2
UNIPROT:Q942N4 Q942N4 Hypothetical protein. 805
1.4e-79 1
UNIPROT:Q9FGM3 Q9FGM3 Protein phosphatase 2C-like. 783
3.0e-77 1
UNIPROT:AAR89521 AAR89521 Hypothetical protein (Fragment). 760
8.2e-75 1
UNIPROT:Q8L7I4 Q8L7I4 At1g78200/T11I11_14. 568
1.8e-54 1
UNIPROT:Q9ZPL8 Q9ZPL8 Protein phosphatase type 2C. 558
2.1e-53 1
UNIPROT:Q94AE3 Q94AE3 At1g78200/T11I11_14. 557
2.7e-53 1
UNIPROT:Q9LME4 Q9LME4 T16E15.10 protein (Hypothetical pro... 545
5.0e-52 1
UNIPROT:Q9S9Z7 Q9S9Z7 F21H2.4 protein (Protein phosphatas... 545
5.0e-52 1
UNIPROT:Q9MAQ8 Q9MAQ8 F28H19.16 protein (Fragment). 286
1.1e-51 2
UNIPROT:Q9SIU8 Q9SIU8 Hypothetical protein (At2g20630/F23... 540
1.7e-51 1
UNIPROT:Q7XPM4 Q7XPM4 OSJNBa0085I10.2 protein. 538
2.7e-51 1
UNIPROT:Q9C9R2 Q9C9R2 Hypothetical protein. 535
5.7e-51 1
UNIPROT:Q8LFF8 Q8LFF8 Protein phosphatase type 2C, putative. 534
7.3e-51 1
UNIPROT:Q93YW5 Q93YW5 Hypothetical protein. 525
6.5e-50 1
UNIPROT:CAE03658 CAE03658 OSJNBa0060N03.23 protein. 524
8.3e-50 1
UNIPROT:Q8H3G4 Q8H3G4 Hypothetical protein. 516
5.9e-49 1
UNIPROT:Q9LDA7 Q9LDA7 Protein phosphatase type 2C (Hypoth... 515
7.5e-49 1
UNIPROT:Q8L834 Q8L834 Hypothetical protein. 511
2.0e-48 1
UNIPROT:Q84QD6 Q84QD6 Avr9/Cf-9 rapidly elicited protein ... 491
2.6e-46 1
UNIPROT:O64583 O64583 Hypothetical protein. 486
8.9e-46 1

UNIPROT:Q7PPP5 Q7PPP5 ENSANGP00000020770 (Fragment). 401
1.4e-45 2
UNIPROT:O49449 O49449 Protein phosphatase 2C-like protein. 482
2.4e-45 1
UNIPROT:Q8C021 Q8C021 CDNA FLJ30553 FIS. 475
1.3e-44 1
UNIPROT:Q8BHN0 Q8BHN0 CDNA FLJ30553 FIS. 475
1.3e-44 1
UNIPROT:Q810H0 Q810H0 Protein phosphatase 2C epsilon. 475
1.3e-44 1
UNIPROT:O24078 O24078 Protein phosphatase 2C. 474
1.7e-44 1
UNIPROT:Q8RX37 Q8RX37 Hypothetical protein. 474
1.7e-44 1
UNIPROT:Q9XEE8 Q9XEE8 Protein phosphatase 2C (AthPP2C5). 473
2.1e-44 1
UNIPROT:O80871 O80871 Hypothetical protein. 469
5.6e-44 1
UNIPROT:Q9SM39 Q9SM39 Putative serine/threonine phosphata... 465
1.5e-43 1
UNIPROT:Q9LMK9 Q9LMK9 F10K1.13 protein. 261
2.2e-42 2
UNIPROT:P2C2_ARATH O04719 Protein phosphatase 2C ABI2 (EC... 369
2.1e-41 3
UNIPROT:AAM97081 AAM97081 Protein phosphatase 2C ABI2. 369
2.1e-41 3
UNIPROT:AAP68299 AAP68299 At5g57050. 369
2.1e-41 3
UNIPROT:Q84JI0 Q84JI0 Putative serine/threonine phosphata... 342
2.1e-41 3
UNIPROT:Q8H6D3 Q8H6D3 Putative serine/threonine phosphata... 434
2.9e-40 1
UNIPROT:Q9SD02 Q9SD02 Protein phosphatase 2C-like protein. 421
6.9e-39 1
UNIPROT:O22200 O22200 Hypothetical protein. 421
6.9e-39 1
UNIPROT:Q940A2 Q940A2 Hypothetical protein. 421
1.8e-38 1
UNIPROT:Q9FLI3 Q9FLI3 Protein phosphatase-2C, PP2C-like p... 327
3.5e-38 3
UNIPROT:Q9CAJ0 Q9CAJ0 Protein phosphatase 2C (AtP2C-HA). 300
3.6e-38 4
UNIPROT:O81709 O81709 Protein phosphatase 2C. 300
3.6e-38 4
UNIPROT:Q8CB81 Q8CB81 Similar to PP2CH (Fragment). 416
5.5e-38 1
UNIPROT:Q80TL0 Q80TL0 MKIAA1072 protein (Fragment). 416
7.0e-38 1
UNIPROT:P2C1_ARATH P49597 Protein phosphatase 2C ABI1 (EC... 362
1.1e-37 2
UNIPROT:AAN13081 AAN13081 Phosphatase ABI1. 362
1.1e-37 2
UNIPROT:Q80Z30 Q80Z30 Calmodulin-dependent protein kinase... 416
1.3e-37 1
UNIPROT:Q9UPT0 Q9UPT0 Hypothetical protein KIAA1072 (Part... 415
1.7e-37 1
UNIPROT:BAA83024 BAA83024 KIAA1072 protein (Fragment). 415
1.7e-37 1

UNIPROT:Q8WY54 Q8WY54 PP2CH. 414
2.3e-37 1
UNIPROT:Q9ZPL9 Q9ZPL9 Nodule-enhanced protein phosphatase... 406
2.7e-37 1
UNIPROT:Q9FIF5 Q9FIF5 Similarity to Ca/calmodulin-depende... 366
3.5e-37 2
UNIPROT:Q944I7 Q944I7 AT5g59220/mnc17_110. 366
3.5e-37 2
UNIPROT:Q8H6D4 Q8H6D4 Putative serine/threonine phosphata... 404
4.3e-37 1
UNIPROT:Q9FRJ1 Q9FRJ1 Hypothetical protein. 403
5.5e-37 1
UNIPROT:Q9FWG2 Q9FWG2 Hypothetical protein. 403
5.5e-37 1
UNIPROT:Q7XCM0 Q7XCM0 Hypothetical protein. 403
5.5e-37 1
UNIPROT:Q7Q4Q8 Q7Q4Q8 AgCP11399 (Fragment). 401
9.0e-37 1
UNIPROT:Q8C1D5 Q8C1D5 CDNA FLJ30553 FIS. 400
1.2e-36 1
UNIPROT:Q9M3V1 Q9M3V1 Protein phpsphatase 2C (PP2C) (EC 3... 361
1.5e-36 2
UNIPROT:P2C4_ARATH P49598 Protein phosphatase 2C (EC 3.1.... 347
2.4e-36 2
UNIPROT:AAL67064 AAL67064 Hypothetical protein. 347
2.4e-36 2
UNIPROT:AAM14330 AAM14330 Hypothetical protein. 347
2.4e-36 2
UNIPROT:Q9LNW3 Q9LNW3 F22G5.22 (Putative phosphatase 2C). 364
3.1e-36 2
UNIPROT:Q8LHG9 Q8LHG9 OSJNBa0062A24.10 protein. 397
8.1e-36 1
UNIPROT:FEM2_HUMAN P49593 Ca(2+)/calmodulin-dependent pro... 390
1.3e-35 1
UNIPROT:BAA02803 BAA02803 KIAA0015 protein (Fragment). 390
1.3e-35 1
UNIPROT:Q7QKC8 Q7QKC8 AgCP14770 (Fragment). 389
1.7e-35 1
UNIPROT:Q8LFK5 Q8LFK5 Protein phosphatase 2C, putative. 357
1.7e-35 2
UNIPROT:P93006 P93006 Hypothetical protein. 388
2.2e-35 1
UNIPROT:Q8GWS8 Q8GWS8 Hypothetical protein. 298
2.4e-35 3
UNIPROT:AAQ03211 AAQ03211 Protein phosphatase 2C. 354
2.7e-35 2
UNIPROT:O82469 O82469 Protein phosphatase-2C. 386
3.5e-35 1
UNIPROT:Q9XZ28 Q9XZ28 BcDNA:LD21794 protein. 385
4.5e-35 1
UNIPROT:FEM2_RAT Q9WVR7 Ca(2+)/calmodulin-dependent prote... 384
5.7e-35 1
UNIPROT:Q84K48 Q84K48 Hypothetical protein. 383
7.3e-35 1
UNIPROT:Q96NM7 Q96NM7 Hypothetical protein FLJ30553. 378
2.5e-34 1
UNIPROT:Q8H610 Q8H610 Putative DNA-binding protein phosph... 378
2.5e-34 1

```
UNIPROT:Q9SLA1 Q9SLA1 Hypothetical protein (Putative phos...    377
3.2e-34   1
UNIPROT:P2C1_SCHPO P40371 Protein phosphatase 2C homolog ...    374
6.5e-34   1
UNIPROT:O82468 O82468 Protein phosphatase-2C.                  344
8.1e-34   2
UNIPROT:Q86A16 Q86A16 Similar to Medicago sativa (Alfalfa...   385
8.4e-34   1
UNIPROT:Q9M1P8 Q9M1P8 Hypothetical protein (Protein phosp...   372
1.1e-33   1
UNIPROT:Q8CGA0 Q8CGA0 Similar to protein phosphatase 1F (...   370
1.7e-33   1
UNIPROT:Q7QVQ7 Q7QVQ7 GLP_302_47488_46331.                     342
2.1e-33   2
UNIPROT:CAF05973 CAF05973 Related to phosphoprotein phosp...   369
2.2e-33   1
UNIPROT:Q9VAK1 Q9VAK1 CG1906 protein.                          366
4.6e-33   1
UNIPROT:Q961C5 Q961C5 LD23542p (CG1906-PE).                    366
4.6e-33   1
```

c) LPL

The translated protein sequence submitted:

SIFYGVRNNALFCRSWFPVYGDLKGLMIIIHGLNVHSGKILQ--ENPGIPCFLFGIHSTGGAVVLKAASCPHIEVMVEGIILTSPALRVKPSIIPIVGAVAPIFSLVAPRFQFKGANKRGIPVSRVPAALLAKYSDPLVYTGPIRVRTGHEILRISSYLMRNFKSVTVPFFVLIIGTADKVTDPLASQDLYNKAASEFKDIKLYDGFLHDLLFEPEREEIAQDIINWMENRLFTSI

BLASTP results:

```
BLASTP 2.0MP-WashU [23-May-2003] [decunix5.0a-ev56-IP32LF64 2003-05-
23T14:40:20]

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USA.
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Reference:  Gish, W. (1996-2003) http://blast.wustl.edu

Query=  Sequence
        (231 letters)

Database:  uniprot
           1,403,392 sequences; 447,949,266 total letters.
Searching....10....20....30....40....50....60....70....80....90....10
0% done


Smallest

Sum

                                                                  High

Probability
```

Sequences producing High-scoring Segment Pairs:			Score
P(N)	N		
UNIPROT:Q9LYG5	Q9LYG5	Lysophospholipase-like protein.	853
1.1e-84	1		
UNIPROT:Q8LEE3	Q8LEE3	Lysophospholipase-like protein.	853
1.1e-84	1		
UNIPROT:Q8S2Q4	Q8S2Q4	Lysophospholipase-like.	839
3.5e-83	1		
UNIPROT:O49147	O49147	Lysophospholipase homolog.	683
2.9e-75	2		
UNIPROT:Q94AM5	Q94AM5	Putative lysophospholipase.	630
1.4e-69	2		
UNIPROT:Q9FX41	Q9FX41	Lysophospholipase homolog, putative.	630
2.6e-68	2		
UNIPROT:Q8VYJ0	Q8VYJ0	At1g18360/F15H18_2.	608
1.3e-66	2		
UNIPROT:Q9LPQ4	Q9LPQ4	F15H18.13.	605
2.2e-58	1		
UNIPROT:Q8LLN8	Q8LLN8	OAJNBa0031O09.10.	470
1.4e-49	2		
UNIPROT:Q9C942	Q9C942	Putative lipase (At1g52760).	248
1.9e-25	2		
UNIPROT:Q8H4S9	Q8H4S9	Putative lysophospholipase isolog.	284
2.3e-24	1		
UNIPROT:Q8RXN7	Q8RXN7	Putative phospholipase.	246
5.6e-24	2		
UNIPROT:O80629	O80629	Putative phospholipase.	246
5.6e-24	2		
UNIPROT:O80628	O80628	Putative phospholipase, alternative...	248
1.2e-23	2		
UNIPROT:Q86A47	Q86A47	Similar to Arabidopsis thaliana (Mo...	235
1.5e-23	2		
UNIPROT:Q8RZT4	Q8RZT4	Phospholipase-like protein.	242
3.0e-23	2		
UNIPROT:Q7ZWC2	Q7ZWC2	Hypothetical protein.	224
4.9e-23	2		
UNIPROT:AAQ97815	AAQ97815	Monoglyceride lipase.	224
4.9e-23	2		
UNIPROT:Q9M3D0	Q9M3D0	Lipase-like protein.	234
1.6e-22	2		
UNIPROT:O04083	O04083	Lysophospholipase isolog, 25331-243...	263
3.8e-22	1		
UNIPROT:O80627	O80627	Putative phospholipase (At2g39400/F...	229
4.2e-22	2		
UNIPROT:Q94D81	Q94D81	B1153F04.5 protein (P0028G04.16 pro...	216
8.0e-22	2		
UNIPROT:Q8U3I6	Q8U3I6	Lysophospholipase.	214
3.7e-21	2		
UNIPROT:Q8F7L2	Q8F7L2	Predicted hydrolases or acyltransfe...	213
3.7e-21	2		
UNIPROT:Q9M3D1	Q9M3D1	Lipase-like protein.	241
8.1e-20	1		
UNIPROT:Q9LF16	Q9LF16	Lipase-like protein.	212
1.2e-19	2		
UNIPROT:AAS06172	AAS06172	Hypothetical protein.	237
2.2e-19	1		
UNIPROT:Q8LGA7	Q8LGA7	Lysophospholipase isolog, putative.	201
2.6e-19	2		

UNIPROT:Q9FVW6 Q9FVW6 Lysophospholipase isolog, putative ... 201
2.6e-19 2
UNIPROT:Q9C5I9 Q9C5I9 Putative lipase. 212
2.9e-19 2
UNIPROT:Q9LFQ7 Q9LFQ7 Lysophospholipase-like protein. 194
3.3e-18 2
UNIPROT:O28521 O28521 Lysophospholipase. 192
3.9e-18 2
UNIPROT:O07427 O07427 POSSIBL oxidoreductase (Lysophospho... 223
6.6e-18 1
UNIPROT:Q7U2M6 Q7U2M6 Possible lysophospholipase (EC 3.1.-). 223
6.6e-18 1
UNIPROT:CAB09734 CAB09734 POSSIBLE LYSOPHOSPHOLIPASE (EC ... 223
6.6e-18 1
UNIPROT:Q9RU57 Q9RU57 Lipase, putative. 222
8.4e-18 1
UNIPROT:Q8LBE6 Q8LBE6 Putative phospholipase. 220
1.4e-17 1
UNIPROT:O22248 O22248 Putative phospholipase (At2g47630/F... 220
2.1e-17 1
UNIPROT:Q96AA5 Q96AA5 Monoglyceride lipase (EC 3.1.1.23). 217
2.8e-17 1
UNIPROT:Q99685 Q99685 Lysophospholipase homolog (Lysophos... 217
2.8e-17 1
UNIPROT:Q9CD03 Q9CD03 Hypothetical protein ML2603. 214
5.9e-17 1
UNIPROT:Q8R431 Q8R431 Monoglyceride lipase. 210
1.6e-16 1
UNIPROT:Q9RRG9 Q9RRG9 Lipase, putative. 209
2.0e-16 1
UNIPROT:O35678 O35678 MONOGLYCERIDE lipase (EC 3.1.1.23). 203
8.6e-16 1
UNIPROT:AAH57965 AAH57965 Mgll protein. 203
8.6e-16 1
UNIPROT:Q8VZV6 Q8VZV6 Putative phospholipase. 179
1.9e-15 2
UNIPROT:Q9AYI2 Q9AYI2 Putative lipase-like protein. 182
2.2e-15 2
UNIPROT:Q7XH23 Q7XH23 Putative lipase-like protein. 182
2.2e-15 2
UNIPROT:Q9UYB4 Q9UYB4 Lysophospholipase. 196
4.8e-15 1
UNIPROT:Q9LZI0 Q9LZI0 Hypothetical protein (Fragment). 196
4.8e-15 1
UNIPROT:Q81KI8 Q81KI8 Hypothetical protein. 172
6.6e-15 2
UNIPROT:Q8YYJ7 Q8YYJ7 Lysophospholipase. 160
2.7e-14 2
UNIPROT:Q8S1R4 Q8S1R4 P0506B12.29 protein. 145
1.2e-13 2
UNIPROT:Q8V549 Q8V549 C5L. 149
8.1e-13 2
UNIPROT:Q8XU47 Q8XU47 Putative lysophospholipase protein ... 148
1.1e-12 2
UNIPROT:AAR07372 AAR07372 13L. 175
1.2e-12 1
UNIPROT:O11456 O11456 H14-E. 148
1.2e-12 2

UNIPROT:YKJ4_YEAST P28321 Hypothetical 35.5 kDa protein i... 176
1.4e-12 1
UNIPROT:Q8F091 Q8F091 Lysophospholipase (EC 3.1.1.5). 160
1.6e-12 2
UNIPROT:CAE50517 CAE50517 Hypothetical protein. 171
3.2e-12 1
UNIPROT:Q8QN19 Q8QN19 V044. 145
3.3e-12 2
UNIPROT:P87627 P87627 M5L protein. 145
3.3e-12 2
UNIPROT:Q816R5 Q816R5 Lysophospholipase L2 (EC 3.1.1.5). 163
6.9e-12 2
UNIPROT:O94305 O94305 Hypothetical protein. 166
9.9e-12 2
UNIPROT:Q944J3 Q944J3 AT3g62860/F26K9_290. 164
1.2e-11 1
UNIPROT:Q8H1P0 Q8H1P0 At3g62860/F26K9_290. 164
1.2e-11 1
UNIPROT:Q8S9W5 Q8S9W5 P0519D04.21 protein. 168
2.6e-11 1
UNIPROT:Q9X171 Q9X171 Lipase, putative. 166
3.6e-11 1
UNIPROT:O34705 O34705 Probable lysophospholipase. 151
1.2e-08 1
UNIPROT:Q7NMI7 Q7NMI7 Gll0778 protein. 152
1.3e-08 1
UNIPROT:Q9K7S1 Q9K7S1 Lysophospholipase. 137
2.1e-08 2
UNIPROT:CAE45873 CAE45873 Hypothetical protein DKFZp686G0... 134
2.6e-08 1
UNIPROT:Q84FD1 Q84FD1 AgmH. 143
1.9e-07 1
UNIPROT:Q8CNU5 Q8CNU5 Lysophospholipase-like protein. 98
1.9e-07 3
UNIPROT:Q99TA3 Q99TA3 SA1584 protein (Lysophospholipase h... 95
3.0e-06 3
UNIPROT:Q7UYF0 Q7UYF0 Lysophospholipase (EC 3.1.1.5). 119
4.1e-06 2
UNIPROT:Q8X9S7 Q8X9S7 Orf, hypothetical protein. 120
5.5e-06 2
UNIPROT:Q8NKD2 Q8NKD2 Lysophospholipase. 134
6.4e-06 1
UNIPROT:Q7RBJ8 Q7RBJ8 Lysophospholipase. 113
6.9e-06 3
UNIPROT:Q8RCK7 Q8RCK7 Lysophospholipase. 105
1.2e-05 3
UNIPROT:YNBC_ECOLI P76092 Hypothetical protein ynbC. 117
1.3e-05 2
UNIPROT:Q871R9 Q871R9 Hypothetical protein 7F4.210. 129
1.4e-05 1
UNIPROT:Q8FHN3 Q8FHN3 Hypothetical protein ynbC. 116
1.7e-05 2
UNIPROT:Q9A2W5 Q9A2W5 Arylesterase-related protein. 130
2.0e-05 1
UNIPROT:Q9QQT4 Q9QQT4 ORFL1R. 107
3.3e-05 1
UNIPROT:Q83KT3 Q83KT3 Orf, conserved hypothetical protein. 115
4.5e-05 2

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UNIPROT:Q9CFB5 Q9CFB5 Hypothetical protein yqaG.                    123
5.4e-05   1
UNIPROT:Q9DHU9 Q9DHU9 13L protein.                                  123
5.9e-05   1
UNIPROT:Q7RQT9 Q7RQT9 Lysophospholipase.                            101
0.00015   3
UNIPROT:Q9KZC3 Q9KZC3 Putative lipase.                              110
0.00015   2
UNIPROT:Q8I2E8 Q8I2E8 Enzyme, putative.                             111
0.00027   2
UNIPROT:CAE80887 CAE80887 InterPro: Esterase/lipase/thioe...        107
0.00056   2
UNIPROT:Q8IC45 Q8IC45 Lysophospholipases-like protein, pu...        102
0.00065   2
UNIPROT:Q7P1P6 Q7P1P6 Probable lysophospholipase L2.                 83
0.00085   3
UNIPROT:Q8ZCZ1 Q8ZCZ1 Hypothetical protein YPO2814.                  101
0.0016    2
UNIPROT:Q8D138 Q8D138 Hypothetical protein.                          101
0.0016    2
UNIPROT:Q81F19 Q81F19 Lysophospholipase L2 (EC 3.1.1.5).             77
0.0020    2
UNIPROT:Q83VA1 Q83VA1 Putative lysophospholipase.                   108
0.0028    1
UNIPROT:CAE78963 CAE78963 Putative Phospholipase/Carboxyl...         88
0.0033    2
UNIPROT:Q897C8 Q897C8 Putative lysophospholipase L2 (EC 3...         69
0.0038    3
```

d) cycMf2

The translated protein sequence submitted to BLAST searching:

EEMCAPRVEEFCFITDNTYTKEEVVKMEKEVLNLLRFQLSVPTTKTFLRR FIQAAQSSYKVPLAELEFLANYLAELTLVEYSFLQFLPSRVAASAVFLARW TLNHSEHPWTTTLEHFTNYKASELKPVVLALEDLQLNTKGCSLHAIREK YKHEKFNGVAKLSPKPVQSLFQAQV

BLASTP results:

```
STP 2.0MP-WashU [23-May-2003] [decunix5.0a-ev56-IP32LF64 2003-05-
23T14:40:20]

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USA.
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Reference:  Gish, W. (1996-2003) http://blast.wustl.edu

Query=  Sequence
        (175 letters)

Database:  uniprot
           1,403,392 sequences; 447,949,266 total letters.
Searching....10....20....30....40....50....60....70....80....90....1
00% done
```

Smallest Sum Probability P(N)	N	Sequences producing High-scoring Segment Pairs:	High Score
8.4e-73	1	UNIPROT:Q39878 Q39878 Mitotic cyclin a2-type.	741
1.6e-64	1	UNIPROT:Q40515 Q40515 A-type cyclin.	663
2.0e-64	1	UNIPROT:Q9XGI4 Q9XGI4 Cyclin A2.	662
1.7e-60	1	UNIPROT:Q39073 Q39073 Cyclin 3b.	625
2.0e-57	1	UNIPROT:Q39071 Q39071 Cyclin 3a.	596
1.8e-56	1	UNIPROT:Q9ZU09 Q9ZU09 Cyclin 3a.	587
9.7e-56	1	UNIPROT:Q39330 Q39330 Cyclin.	580
1.2e-55	1	UNIPROT:Q9S957 Q9S957 Cyclin A homolog.	579
8.7e-55	1	UNIPROT:Q8VYG1 Q8VYG1 Putative cyclin.	571
1.8e-54	1	UNIPROT:Q9XGI0 Q9XGI0 Cyclin A2.	568
2.3e-54	1	UNIPROT:O24073 O24073 Cyclin.	567
6.1e-54	1	UNIPROT:Q9C968 Q9C968 Putative cyclin.	563
1.3e-53	1	UNIPROT:Q9LFM7 Q9LFM7 Cyclin 3b.	560
8.1e-52	1	UNIPROT:Q9M9D7 Q9M9D7 Putative cyclin.	543
2.7e-51	1	UNIPROT:Q941M7 Q941M7 Cyclin A2.	538
1.2e-50	1	UNIPROT:Q38819 Q38819 Cyclin 3c (Fragment).	532
2.5e-50	1	UNIPROT:Q96226 Q96226 Cyclin.	529
2.2e-49	1	UNIPROT:Q38818 Q38818 Cyclin 2.	520
1.4e-45	1	UNIPROT:Q84LE4 Q84LE4 Cyclin A1.	484
1.4e-45	1	UNIPROT:AAR87212 AAR87212 Putative A-type cyclin.	484
1.0e-44	1	UNIPROT:Q40492 Q40492 Cyclin A-like protein.	476
1.2e-43	1	UNIPROT:Q9C6Y3 Q9C6Y3 Mitotic cyclin a2-type, putative.	466
1.9e-43	1	UNIPROT:Q43693 Q43693 Type A-like cyclin.	464
2.4e-43	1	UNIPROT:Q9SSZ6 Q9SSZ6 Cyclin (EST C28985(C63050) CORRESPO...	463

UNIPROT:Q41732 Q41732 Cyclin IIZm (Fragment). 458
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UNIPROT:Q9AVT0 Q9AVT0 Putative A-like cyclin (Fragment). 458
8.2e-43 1
UNIPROT:Q40491 Q40491 Cyclin A-like protein. 453
2.8e-42 1
UNIPROT:Q9XGI5 Q9XGI5 Cyclin A1. 452
3.6e-42 1
UNIPROT:Q40514 Q40514 A-type cyclin. 452
3.6e-42 1
UNIPROT:Q40490 Q40490 Cyclin A-like protein. 452
3.6e-42 1
UNIPROT:Q39879 Q39879 Mitotic cyclin a2-type. 449
7.4e-42 1
UNIPROT:Q9FMH5 Q9FMH5 Cyclin A-type. 448
9.4e-42 1
UNIPROT:O04388 O04388 A-like cyclin. 447
1.2e-41 1
UNIPROT:O04399 O04399 A-type cyclin. 445
2.0e-41 1
UNIPROT:Q40488 Q40488 Cyclin A-like protein. 441
5.2e-41 1
UNIPROT:Q9C6A9 Q9C6A9 Cyclin, putative (At1g47210/F8G22_8). 437
1.4e-40 1
UNIPROT:Q9M4K9 Q9M4K9 Cyclin A3.1. 432
4.7e-40 1
UNIPROT:Q39331 Q39331 Cyclin. 428
1.2e-39 1
UNIPROT:Q9XGI3 Q9XGI3 Cyclin A3. 426
2.0e-39 1
UNIPROT:Q9C6B1 Q9C6B1 Cyclin, putative. 424
3.3e-39 1
UNIPROT:Q39877 Q39877 Mitotic cyclin a1-type. 420
8.7e-39 1
UNIPROT:CG2A_DAUCA P25010 G2/mitotic-specific cyclin C13-... 415
3.0e-38 1
UNIPROT:Q9C6B0 Q9C6B0 Cyclin, putative. 415
3.0e-38 1
UNIPROT:Q8GUV9 Q8GUV9 Cyclin A (Fragment). 396
3.1e-36 1
UNIPROT:Q9FVX0 Q9FVX0 Mitotic cyclin a2-type, putative. 384
5.7e-35 1
UNIPROT:O15996 O15996 Cyclin A. 355
6.8e-32 1
UNIPROT:Q40489 Q40489 Cyclin A-like protein (Fragment). 352
1.4e-31 1
UNIPROT:Q9NIP5 Q9NIP5 Cyclin A. 351
1.8e-31 1
UNIPROT:O62573 O62573 Cyclin A. 319
4.4e-28 1
UNIPROT:Q8LR90 Q8LR90 Putative cyclin Ia. 299
7.5e-26 1
UNIPROT:CG2A_CHLVR P51986 G2/mitotic-specific cyclin A (F... 296
1.2e-25 1
UNIPROT:Q8IY91 Q8IY91 Cyclin A1. 297
1.7e-25 1
UNIPROT:CGA1_HUMAN P78396 Cyclin A1. 297
1.8e-25 1

UNIPROT:CGA2_XENLA P47827 Cyclin A2. 288
8.5e-25 1
UNIPROT:CG2B_SPISO P13952 G2/mitotic-specific cyclin B. 288
9.4e-25 1
UNIPROT:Q98TI4 Q98TI4 Cyclin B3. 286
1.4e-24 1
UNIPROT:CGB3_CHICK P39963 G2/mitotic-specific cyclin B3. 283
2.9e-24 1
UNIPROT:CG1B_MEDVA P46277 G2/mitotic-specific cyclin 1 (B... 284
2.9e-24 1
UNIPROT:Q40337 Q40337 B-like cyclin. 284
2.9e-24 1
UNIPROT:O77211 O77211 Cyclin B. 284
3.3e-24 1
UNIPROT:CGA1_XENLA P18606 Cyclin A1. 282
3.9e-24 1
UNIPROT:CGA1_CARAU Q92161 Cyclin A1 (Cyclin A). 281
4.7e-24 1
UNIPROT:Q9PW42 Q9PW42 Cyclin A. 281
4.7e-24 1
UNIPROT:CGA1_MOUSE Q61456 Cyclin A1. 281
5.6e-24 1
UNIPROT:Q8C5U1 Q8C5U1 Cyclin A1. 281
5.6e-24 1
UNIPROT:Q9XGI1 Q9XGI1 Cyclin B2. 281
7.5e-24 1
UNIPROT:Q7T3L6 Q7T3L6 Cyclin A1. 279
7.6e-24 1
UNIPROT:P90681 P90681 Cyclin A. 281
9.0e-24 1
UNIPROT:CG2B_MEDSA P30278 G2/mitotic-specific cyclin 2 (B... 278
9.8e-24 1
UNIPROT:Q9PW43 Q9PW43 Cyclin A. 277
1.2e-23 1
UNIPROT:CGA2_BOVIN P30274 Cyclin A2 (Cyclin A) (Fragment). 276
1.6e-23 1
UNIPROT:O65064 O65064 Probable G2/mitotic-specific cyclin... 276
1.6e-23 1
UNIPROT:CG2B_MEDVA P46278 G2/mitotic-specific cyclin 2 (B... 278
1.7e-23 1
UNIPROT:CGA2_MESAU P37881 Cyclin A2 (Cyclin A). 277
1.7e-23 1
UNIPROT:Q9FG02 Q9FG02 Mitosis-specific cyclin 1b. 277
2.6e-23 1
UNIPROT:CGB1_CRILO Q08301 G2/mitotic-specific cyclin B1. 275
3.4e-23 1
UNIPROT:Q7XSJ6 Q7XSJ6 OSJNBb0078D11.10 protein. 274
3.7e-23 1
UNIPROT:Q40670 Q40670 Cyclin (Fragment). 271
5.4e-23 1
UNIPROT:Q39067 Q39067 Cyclin. 274
5.7e-23 1
UNIPROT:CGB1_MESAU P37882 G2/mitotic-specific cyclin B1. 273
5.7e-23 1
UNIPROT:CGA2_HUMAN P20248 Cyclin A2 (Cyclin A). 272
8.0e-23 1
UNIPROT:Q9SSZ5 Q9SSZ5 Cyclin. 271
8.4e-23 1

UNIPROT:CGA2_MOUSE P51943 Cyclin A2 (Cyclin A). 271
8.7e-23 1
UNIPROT:Q8BRG1 Q8BRG1 Cyclin A2 (Ccna2 protein). 271
8.7e-23 1
UNIPROT:CG2B_DICDI P42524 G2/mitotic-specific cyclin B. 271
1.1e-22 1
UNIPROT:CG2B_CHLVR P51987 G2/mitotic-specific cyclin B. 268
1.1e-22 1
UNIPROT:CG2A_SPISO P04962 G2/mitotic-specific cyclin A. 269
1.5e-22 1
UNIPROT:Q39880 Q39880 Mitotic cyclin b1-type. 270
1.5e-22 1
UNIPROT:Q9SHP1 Q9SHP1 Putative cyclin 2. 269
1.7e-22 1
UNIPROT:Q39068 Q39068 Cyclin 2a protein. 269
1.7e-22 1
UNIPROT:CG2B_HYDAT P51988 G2/mitotic-specific cyclin B (F... 266
1.8e-22 1
UNIPROT:Q41731 Q41731 Cyclin IIIZm. 268
2.0e-22 1
UNIPROT:CGB1_MOUSE P24860 G2/mitotic-specific cyclin B1. 268
2.2e-22 1
UNIPROT:CGB1_HUMAN P14635 G2/mitotic-specific cyclin B1. 268
2.3e-22 1
UNIPROT:AAP88038 AAP88038 Cyclin B1. 268
2.3e-22 1
UNIPROT:CGB1_RAT P30277 G2/mitotic-specific cyclin B1. 267
2.6e-22 1
UNIPROT:AAH59113 AAH59113 Ccnb1 protein. 267
2.6e-22 1
UNIPROT:Q7ZVJ2 Q7ZVJ2 Hypothetical protein. 266
2.7e-22 1
UNIPROT:CG22_SOYBN P25012 G2/mitotic-specific cyclin S13-... 264
3.0e-22 1
UNIPROT:Q98TA3 Q98TA3 Cyclin A2. 266
3.7e-22 1

Ingredients	$B_5$0	B_5IV	B_5/3M
Maco-elements			
$(NH_4)_2SO_4$	134	134	1,659
KNO_3	2,500	2,500	2,500
$CaCl_2 \cdot 2H_2O$	150	150	150
$MgSO_4 \cdot 7H_2O$	250	250	250
$NaH_2PO_4 \cdot H_2O$	150	150	150
Iron source			
$FeSO_4 \cdot 7H_2O$	27.8	27.8	27.8
Na_2EDTA	33.6	33.6	33.6
Micro-elements			
$MnSO_4 \cdot H_2O$	10	10	10
$ZnSO_4 \cdot 7H_2O$	2.0	2.0	2.0
H_3BO_3	3.0	3.0	3.0
KI	0.75	0.75	0.75
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.25
$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.025
$CoCl_2 \cdot 6H_2O$	0.025	0.025	0.025
Organic supplements			
myo-inositol	500	500	500
nicotinic acid	1.0	1.0	1.0
pyridoxine·HCl	1.0	1.0	1.0
thiamine·HCl	10.0	10.0	10.0
Casein hydrolysate	500	500	500
Sucrose	30,000	30,000	
Maltose			30,000
Adenine		1.0	
Glutathione		10.0	
2,4-D		4.0	
Kinetin		0.2	
PEG6000			25,000
pH	**5.5**	**5.5**	**5.5**

PLANT TISSUE CULTURE MEDIUA (mg L^{-1})

Ingredients	MS	B_5h	Boi2Y
Macro-elements			
NH_4NO_3	1,650		1,000
$(NH_4)_2SO_4$		134	
KNO_3	1,900	3,000	1,000
$Ca(NO_3)_2 \cdot 4H_2O$			500
$CaCl_2 \cdot 2H_2O$	440	1,185	
$MgSO_4 \cdot 7H_2O$	370	500	72
KH_2PO_4	170		300
$Na\ H_2PO_4 \cdot H2O$		150	
KCl			65
Iron source			
$FeSO_4 \cdot 7H_2O$	27.8	27.8	
Na_2EDTA	33.6	33.6	
NaFeEDTA			32.0
Micro-elements			
$MnSO_4 \cdot 4H_2O$	22.3		6.5
$MnSO_4 \cdot H_2O$		10	
$ZnSO_4 \cdot 7H_2O$	8.6	2.0	2.7
H_3BO_3	6.2	3.0	1.6
KI	0.83	0.75	
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	
$CuSO_4 \cdot 5H_2O$	0.025	0.025	
$CoCl_2 \cdot 6H_2O$	0.025	0.025	
Organic supplements			
myo-inositol	100	100	
nicotinic acid	0.5	1.0	0.5
pyridoxine-HCl	0.5	1.0	0.1
thiamine-HCl	0.1	10.0	0.1
glycine	2.0		2.0
Carbon source			
sucrose	30,000	30,000	30,000
Inositol			100
Yeast extract			2,000
L-glutamine		800	
L-glutathione		10	
Adenine		1.0	
Serine		100	
2,4-D		1.0	
Kinetin		0.2	
pH	**5.7**	**5.5**	**5.7**